Published papers supporting the concept

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Report

Low Dose Fractionated Radiation Potentiates the Effects of Taxotere in Nude Mice Xenografts of Squamous Cell Carcinoma of Head and Neck

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hyper-radiation sensitivity, low dose radiation, taxotere, xenografts, chemopotentiation, tumor regression, tumor growth delay.

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ABSTRACT

This study evaluated the combined effect of Low Dose Fractionated Radiation (LDFRT) and Taxotere (TXT) therapy on the growth of SCCHN (squamous cell carcinoma of head and neck; SQ-20B, a p53 mutant SCCHN cell line) tumors in a nude mouse model to exploit the increased hyper radiation sensitivity (HRS) phenomenon present in G_2/M cell cycle phase when induced by low doses of radiation that was demonstrated in in vitro settings. Seventy-eight animals were randomized into one control group and 5 treatment groups (treatments were administered weekly for six weeks). Tumor regression was observed in all the groups, however, tumor regression was not significant in 2 Gy or TXT or 2 Gy plus TXT treated groups when compared to control group. The tumor regression was significant in both the LDFRT group ($p < 0.0043$) and LDFRT + TXT group ($p < 0.0006$) when compared to the other groups. A significantly prolonged tumor growth delay was observed in LDFRT group ($p < 0.0081$). Importantly, in combination of TXT and LDFRT, no tumor regrowth was observed in 12 out of 13 mice since LDFRT + TXT treatment caused a sustained regression of tumors for 9 weeks. Molecular analysis of resected tumor specimens demonstrated that Bax levels were elevated with a concomitant increase in cytochrome c release into the cytosol in treatment Group VI. These findings strongly suggest that LDFRT can be used in combination with TXT to potentiate the effects of drug on tumor regression through an apoptotic mode of death. Furthermore, the G_2/M cell cycle arrest by TXT appears to be an important component of the enhanced apoptotic effect of TXT + LDFRT combined treatment.

INTRODUCTION

Cancer remains the number two cause of mortality and a major public health problem in the United States and in other developed countries. Despite the declining trend in solid tumors of colon, pancreas, stomach and liver, the projected incidence rate of all cancers in the US is estimated at 1,334,100 and resultant deaths from this incidence will be 556,500 for the year 2003.[1] Squamous cell cancer of the head and neck (SCCHN) is a worldwide problem and the third most common cancer among men in developing countries.[2] Women are 2–3 times less likely than men to develop these cancers, particularly in industrialized countries. In the United States, there are approximately 27, 700 new cases of SCCHN projected annually for 2003 with an estimated 7,200 deaths.[1] Nearly 75% of patients present with advanced stage disease,[3,4] and the stage of disease at diagnosis remains the most important survival prognostic factor.[5] Treatment of locally advanced SCCHN involve a combined modality approach, whereas, stage I-II SCCHN is often cured with either surgery or radiotherapy.[5] Historically, the gold standard of treatment for patients with locally advanced SCCHN has been surgery and/or radiotherapy, but more recently, induction chemotherapy and chemo-radiotherapy have been increasingly utilized for locoregional management. Cisplatin and continuous-infusion 5-fluorouracil with or without leucovorin have been most often used as the induction regimen for unresectable locally advanced SCCHN patients[6-9] resulting in higher complete response rates at the expense of increased toxicity.[10-12] Taxanes are effective and promising new agents, particularly docetaxel which has demonstrated significant single-agent activity in recurrent SCCHN.[3,13,14] Docetaxel is similar to paclitaxel with respect to its general mechanism of action (tubulin stabilization and G_2/M cell cycle arrest),[15] however, docetaxel and paclitaxel have somewhat different pharmacodynamics and toxicities that are important in combined regimens. Recurrent or metastatic SCCHN patients treated with docetaxel-cisplatin has shown encouraging response rates and tolerability profiles which has lead to the use of docetaxel regimens as induction therapy for locally advanced disease. Unfortunately, randomized

trials with this treatment approach have not shown improvement in overall patient survival.[16,17] The impact of chemo-resistance is one possible cause of the poor response in advanced stage SCCHN tumors. In addition, radiotherapy toxicities have limited radiation dose escalation and have also resulted in an inability to combine this treatment with full dose chemotherapy. Extensive preclinical research has demonstrated that combined chemotherapy and radiation eliminate malignant cells by the induction of apoptosis as well as by "mitotic death". It is well known that tumor aggressiveness correlates with enhanced resistance to apoptosis. Most tumor cells acquire resistance to therapy through mutation and over-expression of cellular genes that protect against the cell death process. Although, we have an understanding of the mechanism of cell death by radiation at conventional doses (1–2 Gy per fraction), our understanding of radiation effects at lower doses (<1 Gy) is still limited.[18] Until recently, the initial slope of the radiation cell-survival curve (doses of 0–100 cGy) was presumed to be an ineffective dose range for human tumor therapy. However, as techniques to adequately study low dose radiation have improved, quite the opposite effect has been described. Joiner and colleagues revolutionized thinking about low doses of radiation (<100 cGy) by demonstrating an initial phase of hyper radiation sensitivity (HRS) to radiation doses below 1 Gy.[18,19] At doses greater than 1 Gy, increased radiation resistance is found, which is termed "induced-radiation resistance" (IRR) phenomenon. Interestingly, the degree of HRS response varies in different tumor cell lines and this is independent of cell type, apoptosis, radiation-induced cell cycle arrest and p53 functional status.[20-22] The discovery that HRS does not exhibit cellular repair mechanisms that are seen at higher doses provides a plausible explanation as to why there is no induction of radio-resistance with HRS, as measured in vitro.[23] However, as Short and Joiner have asserted, to take advantage of the benefits of low dose fractions radiation in the clinical setting, therapy would have to be extended over 7–12 weeks allowing tumor repopulation, which would abolish the gain due to enhanced cell killing.[24] One alternative to exploit the enhanced cell killing caused by delivering multiple low dose fractions without allowing tumor cell repopulation, would be to combine it with systemic chemotherapy. Specifically, taxanes, a chemotherapeutic class of drugs that arrests the cell cycle at its most radiation sensitive G_2/M phase, representing an exciting option.

We employed a novel strategy combining low dose radiation as an "enhancer" of full dose chemotherapy to circumvent the development of resistance found with standard clinical doses of radiation and chemotherapy. Low dose fractionated radiation (LDFRT) as a chemotherapy potentiator to overcome intrinsic chemotherapy resistance is an intriguing approach to treat both radiation and chemo-resistant tumors. Our recently published in vitro studies demonstrated that LDFRT is a chemo-potentiator of Taxol in two-tumor types viz., head and neck and colorectal cancer.[22,25] The aim of this study was to recapitulate the results of the in vitro data and show that LDFRT can be exploited in combination with taxanes to improve local control of SCCHN SQ20-B xenograft tumors growing in nude mice.

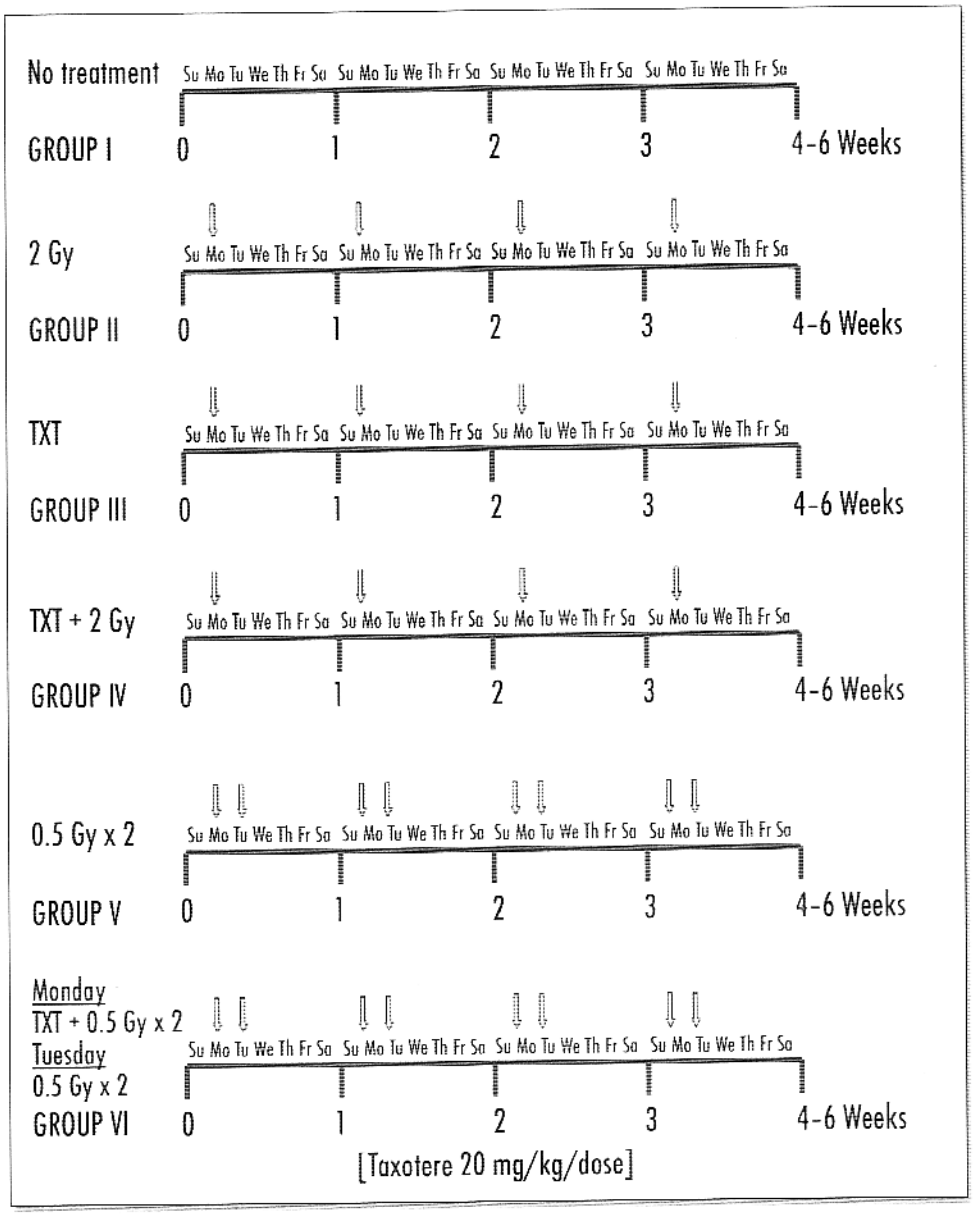

Figure 1. Treatment schedules and groups. Groups V and VI were treated with LDFRT, which was given at 0.5 Gy fractions twice a day for 2 days at the beginning of the week. The docetaxel in group VI was given intra-peritoneally 3 hours prior to the first dose of 0.5 Gy.

MATERIALS AND METHODS

Tumor Cell Line. The tumor cell line SQ20B used in this study is a squamous cell carcinoma of lineage derived from an explant from the oral cavity and is functionally p53 mutant.[22] This tumor line was grown in the laboratory in RPMI medium supplemented with 10% fetal bovine serum,

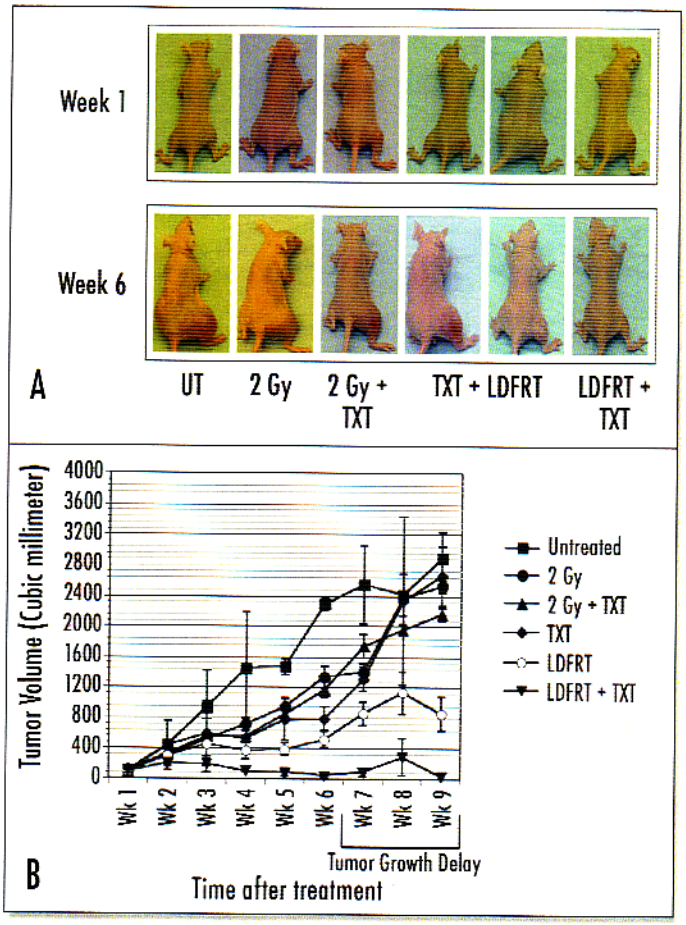

Figure 2. Tumor regression and growth delay in SQ20B xenografts. (A) Nude mice tumor xenografts taken at 1 and 6 week after treatment. (B) Graph showing the tumor volumes of six groups monitored for a period of 9 weeks. The data presented here is a mean of two independent trials with error bars represent standard deviation.

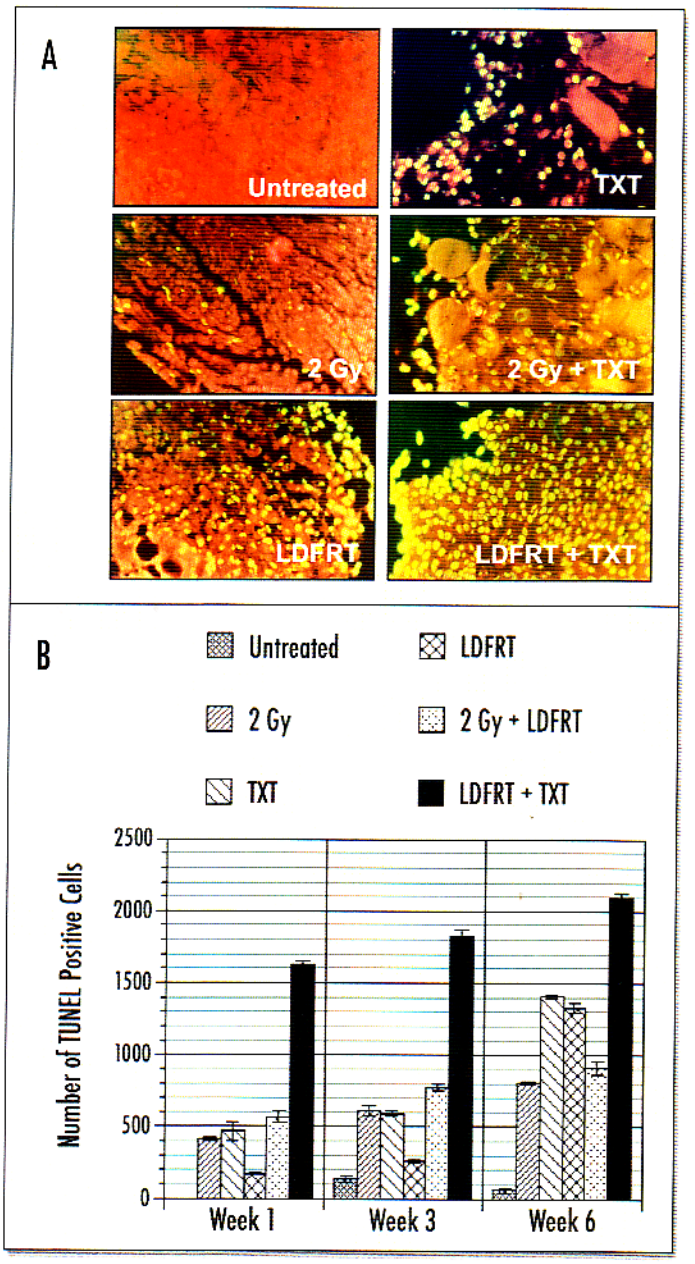

Figure 3. Apoptosis measured in tumor xenografts sampled at week 1, 3 and 6. (A) Micro-photograph of TUNEL positive cells (FITC, yellow) in xenografts obtained after week 1 treatment. (B) A bar graph showing the number of positive TUNEL cells scored among 5000 total cells in a given tumor xenograft specimen obtained from different treatment groups. Error bars represent a mean of TUNEL positive cells from two animals from the same treated group

penicillin-streptomycin and gentamicin. Subcutaneous tumor xenografts were grafted in the flank by injecting of 3–4 million cells per animal.

Mice. Pathogen free athymic Balb/c nude mice, 6–8 weeks old were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice were housed in a pathogen free environment and cared for according to the guidelines stipulated in "Guide for the care and use of laboratory animals", DHHS publication NIH-85-23. SQ20B cells (5×10^6) were directly injected subcutaneously in athymic nude mice on the flank just above the hind leg. The injected mice were monitored twice weekly for the appearance of subcutaneous tumors. The tumor diameters were measured by means of calipers and were assigned to six groups as described in Figure 1, seventy eight (TRIAL I: 30 mice and TRIAL II: 48 mice) mice that received SQ-20B cell injection developed tumors with a minimal treatment tumor volume of ~0.25 cm^3. The tumor volumes were calculated according to the formula of Attia and Weiss: $a^2 \times b \times 0.4$, where "a" is the smallest and "b" the largest diameter of the tumor.[26] The 30 and 48 mice from TRIAL I and TRIAL II respectively, were each distributed in six groups in such a way that the frequency distribution of tumor volumes in each group reflects the distribution of the population from which the groups were formed.

Treatment Schedules and Tumor Tissue Harvest. The nude mice study consisted of six groups. In Trial I and II, 5 and 8 animals were studied in each group respectively. Each group is described in Figure 1 and the treatment schedule; dose of radiation and Taxotere is described below:

Group I: No treatment.

Group II: 2 Gy, once a week and continued for six weeks

Group III: Taxotere, once a week and continued for six weeks.

Group IV: 2 Gy plus Taxotere, once a week and continued for six weeks.

Group V: LDFRT alone (four fractions of 0.5 Gy with 8 hr interval between each fraction), once a week and continued for six weeks.

Group VI: LDFRT plus Taxotere (four fractions of 0.5 Gy with 8 hr interval between each fraction), once a week and continued for six weeks.

Prior to treatment, the mice were tattooed on the tail or ear clipped to enable the follow up of individual animals. The tumors were irradiated locally at room temperature using a ^{137}Cs unit (Mark I irradiator Model 30 with a 4,000 Ci source, Shephard JL, San Fernando, CA) that is installed in our animal facility. The current dose rate is ~5.0 Gy/min at the center of the tumor. A uniform dose (± 2%) can be obtained in the center of an 8 cm length. A 5 cm thick Cerro-band block shielded the whole body of the animal

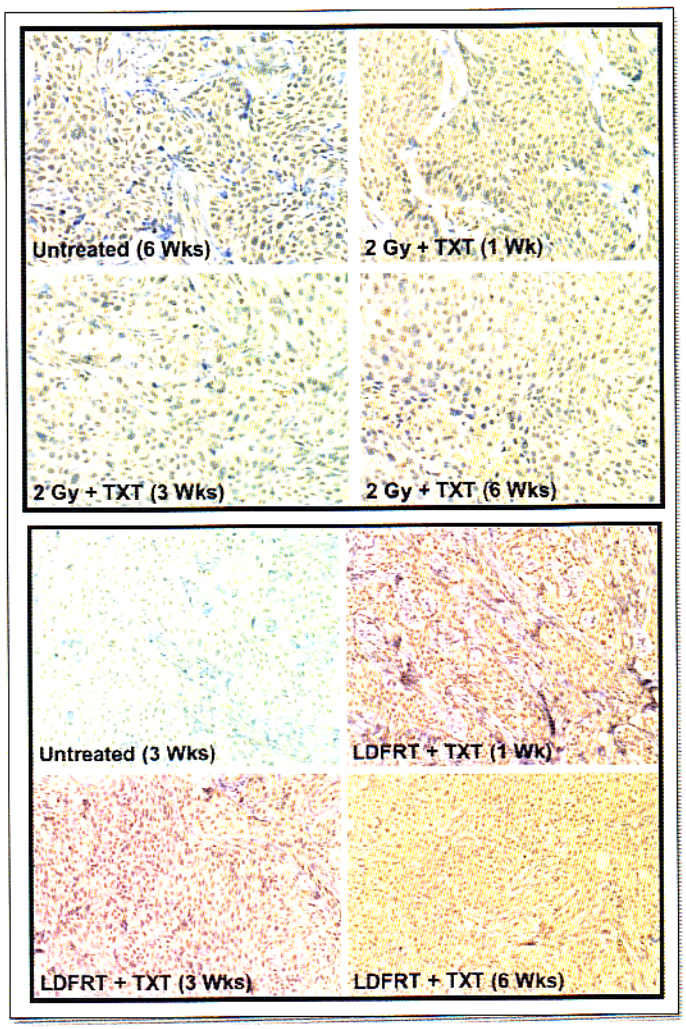

Figure 4. Bax protein expression staining by immunohistochemistry in tumor xenografts obtained at week 1, 3 and 6 from 2 Gy plus TXT or LDFRT plus TXT groups.

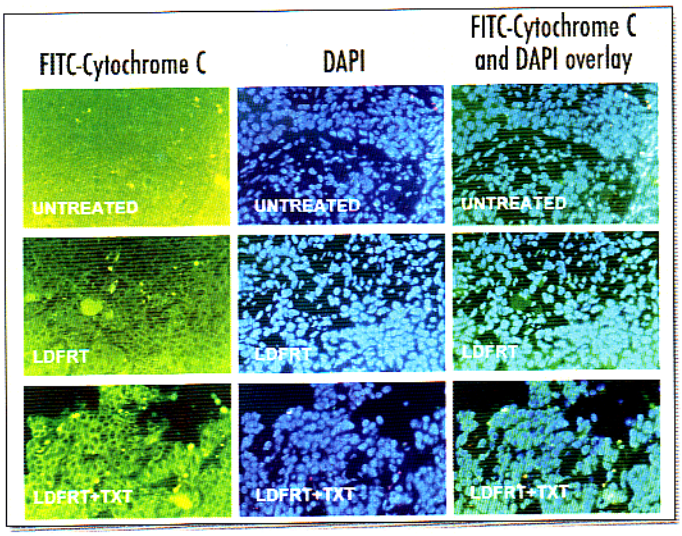

Figure 5. Immunofluorescence of Cytochrome c release in cytosol of tumor xenografts obtained at week 1, 3 and 6 from LDFRT or LDFRT plus TXT groups. The nuclei are stained with DAPI and overlay with DAPI and FITC is shown.

and only the side of the thigh with the tumor was exposed to radiation. Taxotere at a clinically dose of 20mg/kg body was injected intra-peritoneally 3 hours prior to radiation treatment. Tumor volumes were determined twice a week. The mice were checked for signs of general malaise such as: poor grooming, lethargy and cachexia (measured by weight determination). Upon occurrence of these signs, the mice were considered moribund and sacrificed by cervical dislocation following CO_2 inhalation.

Two mice in each group (from Trial II) were euthanized on the 1st, 3rd and 6th week 3 hrs after the last treatment as scheduled for the tumor tissue harvest. These tissues were fixed in 5% buffered formalin and embedded in paraffin. Paraffin-embedded tumor sections were subjected to apoptosis staining by TUNEL assay; immuno-histochemistry staining for Bcl-2 and Bax; and immuno-fluorescence staining for cytochrome C release.

Apoptosis Detection by TUNEL Assay. To determine the incidence of apoptosis in tumor specimens harvested during the course of treatment, the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD), that detects DNA strand breaks by terminal transferase-mediated dUTP- digoxigenin nick end labeling (TUNEL) was used as described.[27] Briefly, the samples were de-paraffinized on slide warmer at 60°C for 5 minutes, then were dipped in xylene for 5 minutes and were further rehydrated by decreasing percent alcohol changes. The DNA in dehydrated tissue section was tailed with digoxigenin-dUTP and conjugated with an anti-digoxigenin fluorescein. The specimen was counter stained with propidium iodide and antifade. The stained specimen was observed in triple band-pass filter using Nikon-microphot epi-fluorescence microscope. Slides were scored for the presence of TUNEL positive cells in two tumor specimens obtained from two different mice of the same treatment group.

Immuno-Histochemistry (IHC). Sections from each treated tumor specimen were stained with the following antibodies: anti-Bax antibody (sc-7480) and anti-Bcl-2 antibody (Santa Cruz Biotechnology, CA). The avidin-biotin-peroxidase complex method of staining was used for immuno-histochemical staining.[28] The IHC assay was scored in a semi-quantitative approach utilizing both the intensity and distribution of specific staining. The staining intensity (I) was graded as 0 (if no staining was observed), 1 (weak staining), 2 (moderate staining) or 3 (strong staining). The proportion (P) of cells (0–1.0) with the observed staining intensity was recorded. A score (so called histologic or H-score) for each case was determined as the product of intensity and proportion (H = I x P).[28]

Immuno-Fluorescence. The tumor sections were de-paraffinized in slide warmer and xylene. Further, these sections were rehydrated in decreasing percent alcohol changes. Antigen unmasking was performed using trypsin. Subsequently, sections were blocked using 10% normal serum in PBS to suppress nonspecific binding of IgG. After PBS washes, the tissue sections were incubated with anti-cytochrome C antibody for 1 hour at 37°C. Slides were washed in PBS and incubated in anti-sheep IgG-FITC secondary antibody for 45 min at 37°C. The slides were mounted with DAPI/Antifade. Presence of Cytochrome C in the cytoplasm was visualized in FITC filter or triple-band pass filter using Nikon-microphot epi-fluorescence microscope.

Statistical Analysis. Means and standard deviations were determined from the data obtained from the two separate trials. Apoptosis, immuno-histochemistry and immuno-fluorescence assays were performed in at least two animal tumor tissue samples from each group and scored individually to obtain the means and standard deviations. Groups were compared using the two-tailed Fisher's exact test.

RESULTS

Low Dose Fractionated Radiation in Combination with Taxotere Conferred Enhanced Tumor Regression on SQ-20B Tumor Xenografts in Nude Mice. In our previous reports using colorectal and SCCHN tumor cell lines, we demonstrated that low doses of radiation (dose that induce HRS) potentiated the effect of Paclitaxel.[22,25] To understand the effect in vivo, SQ-20B xenografts in nude mice were treated with low dose fractionated radiation alone or in combination with Taxotere and regression responses were compared to groups that were left untreated or treated with 2 Gy/ TXT

Table 1 **Correlation Between Growth and Apoptosis with Gene Expression Kinetics of Bcl-2 and Bax in SQ20B Xenografts**

Treatments	Tumor Growth	Bcl-2 Expression	Bax Expression	Cytochrome C Release	TUNEL Positive Cells
No treatment—3rd week	1389	Negative	0.5	Negative	130 ± 23.8
No treatment—6th week	2518	Negative	1	Negative	62 ± 15.2
2 Gy—1st week	185	Negative	1	Weak	412 ± 15.8
2 Gy—3rd week	965	Weak	1	Weak	608 ± 31.2
2 Gy—6th week	1195	Weak	1	Weak	808 ± 8.7
TXT—1st week	408	Moderate	1	Weak	465 ± 62.4
TXT—3rd week	512	Negative	1	Weak	590 ± 15.9
TXT—6th week	1420	Negative	1	Weak	1405 ± 8.6
LDFRT—1st week	246	Weak	1.5	Moderate	175 ± 8.9
LDFRT—3rd week	309	Weak	2	Moderate	261 + 9.9
LDFRT—6th week	863	Weak	2	Moderate	1326 + 30.4
2 Gy + TXT—1st week	256	Negative	1	Weak	568 ± 40.5
2 Gy + TXT—3rd week	491	Moderate	1	Weak	773 ± 22
2 Gy + TXT—6th week	1775	Moderate	1.5	Weak	911 ± 44.5
LDFRT + TXT—1st week	128	Negative	1.5	High	1623 ± 29
LDFRT + TXT—3rd week	10	Negative	1.5	High	1828 ± 40.3
LDFRT + TXT—6th week	3	Negative	2	High	2089 ± 40.4

alone or 2 Gy combined with TXT. Tumor regression was observed in all the treated groups. However, no significant tumor regression was observed in 2 Gy or TXT or 2 Gy plus TXT treated groups when compared to untreated group. On the other hand, the tumor regression was significant in both the LDFRT group ($p < 0.0043$) and LDFRT + TXT group ($p < 0.0006$) when compared to Groups II, III and IV (Fig. 2, Table 1). Accelerated reappearance of tumor growth was evident in groups II, III and IV after six weeks of treatment. By week 8, the tumor volumes of groups II, III and IV equaled those of the untreated group (Fig. 2B). A significantly prolonged tumor growth delay was observed in LDFRT group ($p < 0.0081$). Interestingly, in Group VI, no tumor regrowth was observed in 12 out of 13 mice since LDFRT + TXT treatment caused a sustained regression of tumors till 9 weeks. However, one mouse in this group showed recurrence of tumor growth from week 8 (Fig. 2B). In particular, 4 out 13 mice of Group VI (from Trial I and II) had a complete tumor response with no evidence of disease (that was monitored more than 90 days after treatment, Fig. 2A). These findings corroborate our previously reported in vitro data[22,25] and establish that LDFRT potentiates the effect of Taxotere by causing tumor regression in SQ-20B subcutaneous tumor xenografts in nude mice.

Tumor Regression Is Associated with Increased Apoptosis in LDFRT Plus Taxotere Treated Group. Our previous in vitro studies showed that LDFRT greatly potentiated the effects of Paclitaxel-induced apoptosis in mutant p53 SQ-20B cells when compared to wild-type p53 SCC-61 cells.[22] The apoptotic enhancement ratio for chemo-potentiation was significantly higher than that of the clonogenic inhibition enhancement ratio for LDFRT.[22] These observations indicate that the primary effect of LDFRT + Paclitaxel treatment is mediated through apoptosis. To further understand the mode of regression in SQ-20B sub-cutaneous xenografts, tumor specimens were harvested in each treatment group three hours after the last treatment in weeks 1, 3 and 6. Tissue specimens from week 1 and 3 showed significantly increased apoptosis in LDFRT + TXT group ($p < 0.0023$) when compared to other groups (Fig. 3A and B, Table 1). However, in week 6, a modest increase in cell death was observed in mice treated with TXT alone or LDFRT alone when compared to 2 Gy alone or 2 Gy + TXT (Fig. 3B, Table 1). The sampling of tissue performed at the end of week 6 treatment was small due to significant regression in this group. A majority of the areas in the tissue sample showed TUNEL positive cells (Fig. 3A). Thus, the LDFRT + TXT group showed a sustained significant increase in cell death in the 6th week, suggesting that the enhanced tumor growth regression observed in this group is a result of tumor cells that are eliminated primarily through a cell death mode.

Upregulation of Bax Protein in Xenografts Treated with LDFRT + TXT. In our previous report,[22] significant induction of Bax was observed when Paclitaxel was combined with LDFRT, and this was evident with each 0.5 Gy fraction. Bax induction was found in both p53 efficient as well as p53 mutant cells. To understand the ratio of gene kinetics of pro-apoptotic Bax and pro-survival Bcl-2 in an in vivo tumor environment, we analyzed the expression of Bcl-2 and Bax proteins in the tumor specimens that were harvested in each treatment group three hours after the last treatment in week 1, 3 and 6. Weak to moderate focal induction of Bcl-2 protein was observed in groups II, III, IV and V when compared to complete absence of Bcl-2 expression in the untreated group (Table 1). Significant induction of Bax protein was observed both in LDFRT ($p < 0.005$) and LDFRT + TXT ($p < 0.04$) groups (Table 1, Fig. 4) at weeks 3 and 6. These findings confirm our previously reported in vitro data[22] in which induction of Bax protein upregulation was significant in LDFRT plus taxane group.

Increased Apoptosis and Bax Induction are Associated with Enhanced Cytochrome c Release in LDFRT Plus Taxotere Treated Group. Bax upregulation affects mitochondrial permeability, favoring the release of cytochrome c, leading to caspase-9 activation, which subsequently induces caspase-3 activation[29] and leads to apoptosis. Since LDFRT + TXT treated SQ20-B xenografts showed an increase in the level of Bax protein (Fig. 4), we further characterized the downstream event of cell death by analyzing the cytochrome c release in the tumor specimens that were harvested in each treatment group three hours after the last treatment in weeks 1, 3 and 6. In 2 Gy, TXT and 2 Gy + TXT treated groups, weak release of cytochrome c in the cytosol (sparse punctuated staining) was observed (Fig. 5, Table 1) when compared with control group; whereas, in LDFRT treated group, a moderate increase in cytochrome c release in the cytosol was observed (Fig. 5, Table 1). Furthermore, the punctuated staining of cytochrome c release was highly intense in LDFRT + TXT treated group in week 1, 3 and 6 (Fig. 5, Table 1). These observations show that combined LDFRT + TXT is a positive effector of apoptosis through Bax and cytochrome c release.

DISCUSSION

Extensive data have emerged on the HRS/IRR phenomena observed in more than 40 tumor cell lines in response to single low dose radiation. Studies have also shown that HRS occurs after fractionated low doses in in vitro.[20,30] In a recent report, the effect of low dose ultra-fractionation schedule (0.4 Gy per fraction-3 fraction per day for 21 days, an approach to exploit the HRS phenomenon) was compared with conventional fraction schedule (1.68 Gy per fraction, 1 fraction per day and 5 fractions per week) in response to local control improvement of glioma xenograft tumors.[31] It was found that the low-dose ultra-fractionation caused a significant decrease in tumor growth delay but increased the top-up TCD_{50} dose and further failed to prove the existence of HRS in in vivo.[32] It was not clear from the results of this report that there existed any potential utility of low-dose ultra-fractionation in translating to clinical practice.[31] However, in another recent report, repeated irradiation with low dose (0.8 Gy 3 times / day for 4 days/week to a total of 2 consecutive weeks) was markedly more effective than irradiation with single conventional dose (2 Gy / day, for 4 days/week to a total of 2 consecutive weeks) in inhibiting the tumor xenograft in mice.[33] The authors implied that the ultra-fractionation as apposed to continuous hyperfractionation accelerated radiotherapy, reduced long-term injuries and prevented tumor repopulation in radioresistant tumors.[33] These reported findings are in concordance with results observed in our study where the LDFRT group showed a significant inhibitory effect and a prolonged tumor regrowth delay when compared with 2 Gy group (Fig. 2A and B). Thus, a thorough evaluation of novel treatment options in animal models remains an essential requirement for clinical translation of the HRS phenomenon. The unique significance of the data generated in this study is that LDFRT in combination with TXT significantly controlled tumors of SQ20B xenografts with no tumor regrowth; and remarkably, 30% of animals showed complete tumor cure. This observation is in agreement with our previous findings reported using tumor cell lines which demonstrated the chemo-potentiating effect of low-dose fractionated radiation.[22,25] Together, our in vivo and in vitro studies demonstrate that LDFRT can be exploited to enhance the effect of chemotherapy for improved killing of tumor cells. Thus, the approach of combining low-dose fractionated radiation with chemotherapy establishes a platform for clinical translation of the HRS phenomenon.

Recently, Short et al. demonstrated the influence of cell cycle phases on the HRS phenomenon. Cells synchronized in G_2/M showed a significantly more pronounced HRS than synchronized cells in G_1 or S.[34] The finding of Short et al. presents an interesting explanation of the underlying mechanism of chemo-potentiation by LDFRT in this study. In this study, TXT was given prior to LDFRT which is also known to cause G_2/M arrest.[35] When these tumor cells are in the process of undergoing G_2/M arrest, four fractions of 0.5 Gy are delivered. Thus, it can be conceived that the effect of low-dose fractions on G_2/M arrested cells (since pronounced induction of HRS occur in G_2/M phase) contributed to the significantly enhanced killing and regression of tumors in LDFRT + TXT group.

Our previous in vitro study demonstrated increased cell death rather than clonogenic inhibition in LDFRT mediated chemo-potentiation.[22] As seen in Figure 3B, our in vivo data suggest a significant increase in cell death observed starting from weeks 1, 3 and 6. This suggests that the chemo-potentiation by LDFRT kills tumor cells primarily through the apoptotic mode. The induction of BAX protein is the notable similarity observed in the process of cell death in both our in vitro as well as in vivo studies. It has been reported that low doses of radiation result in apoptotic cell death in invasive squamous cell carcinoma in association with the increased expression of Bax but not with increased Bcl-2 expression.[36] Ohno et al.[37] reported that in cervical tumors, a significant correlation between Bax protein expression and apoptosis positivity was observed after administering 9 Gy total dose given as fractionated doses. These findings appear to support the data presented here and indicate that Bax protein induction is associated with apoptosis induced by low-dose fractionated radiation therapy.

Previously, we reported that a single fraction of 2 Gy dose caused induction of NFκB activity and Bcl-2 expression in SCCHN tumor cell lines, whereas, LDFRT caused induction of Bax with downregulation of Bcl-2 expression and NFκB activity.[22] We posited that these signaling events induced by LDFRT may have led to chemo-potentiating effect of Paclitaxel in SCCHN cells. As observed in the in vitro study, we found similar induction of Bax, as well as cytochrome C release in this in vivo study. Thus, this signaling pathway might play a pivotal role in LDFRT mediated chemo-potentiating effect. As stated earlier, Paclitaxel mediated G_2/M arrest are conducive for LDFRT to maximally exert HRS mediated killing. In addition, we recently found that 2 Gy or 7 Gy single fraction induced MDR-1 expression (multi-drug resistant-1 or P-Glycoprotein), whereas, LDFRT failed to induce MDR-1 gene expression (data not shown). Recently, it has been shown that activators of NFκB cause induction of MDR-1 gene expression.[38] Thus, we have theorized that lack of induction of MDR-1 gene expression in LDFRT treatment might be due to absence of NFκB induction, whereas, the presence of MDR-1 gene induction in 2 Gy single fraction is due to induction of NFκB activity. Thus, the lack of MDR-1 gene expression in LDFRT group may be an important step rendering the chemo-potentiating effect for Taxotere. In summary, the potentiation by LDFRT on the Taxotere-induced apoptosis of SCCHN cells can be achieved by three different mechanisms. They are (1) significant induction of Bax with lack of upregulation of NFκB activity and Bcl-2 expression; (2) LDFRT compounds the HRS effect in Taxoteremediated G_2/M cell cycle block; and (3) lack of upregulation of MDR-1 gene by LDFRT.

The findings from both in vitro and in vivo studies have led us to utilize the HRS phenomenon in the clinical setting. Based on demonstrated synergy between Taxol and LDFRT, our group designed a novel neo-adjuvant therapy utilizing LDFRT in combination with Carboplatin/Paclitaxel for patients with locally advanced stage III and IV SCCHN. This novel approach provided a response rate (RR) of 90% at the primary site and a nodal RR of 69%.[39] LDFRT combined with paclitaxel and carboplatin was effective in SCCHN and has a similar toxicity profile to chemotherapy alone. Together, (i) the data obtained from our previously published in vitro study,[22] (ii) the findings of this in vivo study and (iii) the clinical study[39] strongly suggest that the use of low dose radiation in multiple fractions with a chemotherapeutic agent that harness its G_2/M block function like Paclitaxel, is a novel approach to exploit the observed HRS phenomenon in order to achieve significant chemo-potentiation, eliminate IRR and improve overall survival in solid tumors.

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Low Dose Fractionated Radiation Enhances the Radiosensitization Effect of Paclitaxel in Colorectal Tumor Cells with Mutant p53

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BACKGROUND. The current study was undertaken to investigate the influence of wild-type or mutant p53 status on the radiosensitizing effect of paclitaxel in colorectal tumor cell lines.

METHODS. HCT-116 (contains wild-type p53) and HT-29 (contains mutant p53) established from moderately differentiated colorectal carcinomas were used in this study. Colony-forming assay was performed after exposure to either different radiation doses (0.5–6 gray [Gy]) or paclitaxel (1–10 nM) or in combination. Induction of p53 and $p21^{waf1/cip1}$ by these treatments were determined by immunocytochemistry and Western blot analysis.

RESULTS. Radiation caused an increase in nuclear p53 and $p21^{waf1/cip1}$ proteins in HCT-116 cells, indicating that p53 functionally induced $p21^{waf1/cip1}$. However, induction of nuclear p53 and $p21^{waf1/cip1}$ protein was not evident in HT-29 cells, suggesting that p53 was not functional in these cells. Survival data showed that the HCT-116 cells (survival fraction of exponentially growing cells that were irradiated at the clinically relevant dose of 2 Gy [SF_2] = 0.383; dose required to reduce the fraction of cells to 37% [D_0] = 223 centigray [cGy]) were significantly sensitive to ionizing radiation ($P < 0.008$) when compared with the HT-29 cells (SF_2 = 0.614; D_0 = 351 cGy). Paclitaxel caused a higher degree of clonogenic inhibition in HCT-116 (D_0 = 0.7 nM) than HT-29 (D_0 = 1.11 nM) cells ($P < 0.06$). When paclitaxel and radiation were combined, an enhanced radiosensitizing effect ($P < 0.05$) was observed in HCT-116 cells (SF_2 = 0.138; D_0 = 103 cGy), whereas in HT-29 cells no significant radiosensitization of paclitaxel was observed (SF_2 = 0.608; D_0 = 306 cGy). However, pretreatment with paclitaxel followed by multifractionated low dose radiation (0.5- or 1-Gy fractions for a total dose of 2 Gy) significantly enhanced the radiosensitizing effect in both HCT-116 and HT-29 cells.

CONCLUSIONS. The results of the current study suggested that multifractionated radiation given at very low doses after exposure of cells to paclitaxel conferred a potent radiation sensitizing effect irrespective of p53 status. ***Cancer* 2000;89: 1893–900.** © *2000 American Cancer Society.*

KEYWORDS: paclitaxel, fractionated radiation, p53, colorectal tumors, radiosensitization.

Colorectal carcinoma is the fourth most diagnosed malignancy as well as the second most common cause of cancer death in the U.S. On an annual basis approximately 129,400 new cases are diagnosed and approximately 47,900 individuals die from colorectal carcinoma.[1] Surgery is the primary treatment and results in cure in approximately 58% of patients.[2] Radiotherapy either given before surgery or after has been shown to lower the local recurrence rate up to 50% in some studies.[2] 5-fluorouracil plus leucovorin has for many years been the

standard treatment of patients with metastatic colorectal carcinoma. The overall 5-year survival rate of colorectal carcinoma is limited by depth of tumor invasion and histopathologic type.[2]

One of the molecular determinants regulating the response to ionizing radiation is the tumor suppressor protein p53, which serves as a pivotal component of the apoptosis pathway(s) in diverse cell types. Wild-type p53 protein confers radiation responsiveness, which causes either G_1 cell cycle arrest and/or apoptotic death, this effect is mediated by activation of other downstream target genes such as $p21^{waf1/cip1}$.[3,4] The p53 protein may be an important determinant of cellular sensitivity to anticancer agents, including paclitaxel.[5,6] Paclitaxel is a microtubule stabilizing agent effective for cancer therapy against ovarian carcinoma, breast carcinoma, malignant melanoma, myoblastic leukemia, and other carcinomas.[7,8] Paclitaxel effectively blocks exponentially growing cells in the G_2/M-phase, the most radiosensitive phase of the cell cycle.[9] To our knowledge the increased sensitivity to paclitaxel in association with p53 abrogation has not been observed consistently. Correlation between p53 and paclitaxel sensitivity was not found in ovarian carcinoma cells.[10] Several human xenografts that responded to paclitaxel in preclinical trials were found to have p53 mutations.[11–13] However, paclitaxel-induced cell cycle arrest is compromised in murine fibroblasts lacking p53, suggesting that p53 may contribute to the biologic effects of paclitaxel.[14] Because p53 protein is mutated and nonfunctional in a large number of colorectal tumors,[15] it is important to identify other novel approaches that can function via a p53 independent mechanism for the containment of radioresistant colorectal tumors. The impact of p53 disruption on the sensitivity of mammalian cells to DNA-damaging agents has received much attention. Paclitaxel has the potential to improve radiotherapy and in some cell lines paclitaxel has been shown to enhance the radiation response.[16] Therefore, the current study was undertaken to investigate the influence of wild-type and mutant p53 status on the radiosensitization effects of paclitaxel in colorectal tumor cell lines that were irradiated, either with single or multiple radiation dose fractions.

MATERIALS AND METHODS

Cell Lines

Two established colorectal carcinoma cell lines (HCT-116 and HT-29) were obtained from American Type Culture Collection (Rockville, MD). The HCT-116 cell line was established from a carcinoma of the colon obtained from a male patient and these cells were tumorigenic.[17] The HT-29 cell line was established from an adenocarcinoma of the colon obtained from a 44-year-old female.[18,19] In addition, HT-29 cells are highly tumorigenic and these tumors were well differentiated adenocarcinomas consistent with colonic primary (Grade 1) tumors.[20] Unlike other tumor cell lines that commonly are established from metastatic tumor tissue, the HCT-116 and HT-29 cell lines were established directly from a primary colon tumor. Thus, HCT-116 and HT-29 cells form a precise representation of human colon carcinoma. HCT-116 contains wild-type p53,[21] whereas HT-29 contains mutant p53.[22] HCT-116 was grown in Dulbecco modified Eagle medium with high glucose, 10% fetal calf serum, and 1% antibiotics. HT-29 cells were grown in McCoys 5A medium with 10% fetal calf serum and 1% antibiotics.

Irradiation

A 100-kilovolt industrial X-ray machine (Philips, Hamburg, Germany) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm aluminum plus 1-mm beryllium filter was approximately 1.85 Gy/minute at a focus-surface distance of 30 cm.

Immunocytochemistry

Expression of p53 and $p21^{waf1/cip1}$ was determined by immunocytochemical analysis. Cells were exposed to different doses of radiation, paclitaxel, or a combination of the two. Treated cells were incubated for different lengths of time at 37 °C and were subjected to immunocytochemistry with the anti-p53 antibody (DO-1) and anti-$p21^{waf1/cip1}$ antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). As per the instructions in the Vectastain Elite ABC Kit manual (Vector Laboratories, Burlingame, CA), reactions with biotinylated antirabbit immunoglobulin antibody, and avidin-biotin-peroxidase complexes and staining with diaminobenzidine and hydrogen peroxide were then performed sequentially.

Western Blot Analysis

Total protein extracts from untreated and irradiated cells at various time intervals were subjected to Western blot analysis using the anti-p53 antibody, anti-$p21^{waf1/cip1}$ antibody, or β-actin antibody (Sigma Chemical Company, St. Louis, MO) for a loading control as described previously.[23]

Colony–Forming Assay

For clonogenic cell survival studies, two different cell concentrations in quadruplet sets were used for each radiation dose. Cell lines were left untreated or exposed to 0.5–6 Gy of radiation alone or to different concentrations of paclitaxel (Taxol®; Bristol-Myers

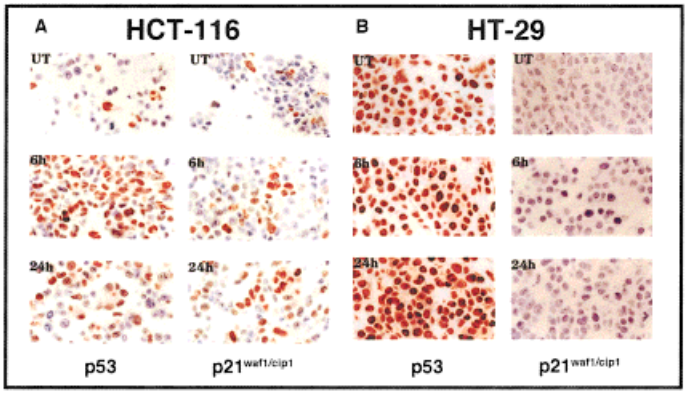

FIGURE 1. Immunocytochemical analysis of nuclear p53 and $p21^{waf1/cip1}$ induction in (A) HCT-116 and (B) HT-29 cells. Cells were left untreated or were irradiated and subjected to immunocytochemistry using anti-p53 antibody (DO-1) and anti-p21 antibody.

Squibb Oncology, Princeton, NJ). For combined experiments, the cells first were treated with paclitaxel (1 nM) and irradiated in the same medium with different doses of radiation after 24 hours. After incubation for $\geq$ 10 days, each flask was stained with crystal violet and the colonies containing $>$ 50 cells were counted. The surviving fraction (SF) was calculated as a ratio of the number of colonies formed and the product of the number of cells plated and the plating efficiency. The curve was plotted using X-Y log scatter (Delta Graph® 4.0; Delta Point, Inc.) and by using the formula of the single hit multitarget model; the D_0 was calculated as described previously.[23] D_0 is the dose required to reduce the SF of the cells to 37%, indicative of single event killing. SF_2 is the SF of exponentially growing cells that were irradiated at the clinically relevant dose of 2 Gy. The radiation enhancement ratio (ER) for paclitaxel was calculated using the formula:

$$ER = \text{Mean of } SF_2ER + D_0ER, \text{ in which } SF_2ER = \frac{SF_2 \text{ of radiation alone}}{SF_2 \text{ of radiation + paclitaxel}}$$

and

$$D_0ER = \frac{D_0 \text{ of radiation alone}}{D_0 \text{ of radiation + paclitaxel}}$$

For multifractionated experiments, cells were exposed to 1 nM of paclitaxel and 24 hours later were irradiated in the same medium to 0.5- or 1-Gy fractions for a total dose of 2 Gy with an 8-hour time delay between each fraction. Colony–forming ability at these doses for the total dose of 2 Gy (SF_2) was calculated. ER was calculated using SF_2 values for radiation alone and radiation plus paclitaxel treatment. An ER $>$ 1 indicated radiosensitization by paclitaxel.

RESULTS

Radiation Elevates p53 and $p21^{waf1/cip1}$ in HCT-116 Cells

To ascertain whether radiation causes elevation of nuclear p53 and $p21^{waf1/cip1}$ protein, cells were treated with different concentrations of paclitaxel and/or radiation and immunocytochemistry was performed after various time intervals using anti-p53 and anti-$p21^{waf1/cip1}$ antibodies. Radiation caused an increase in nuclear p53 protein and $p21^{waf1/cip1}$ in HCT-116 cells (Fig. 1A). However, radioinduction of nuclear p53 and $p21^{waf1/cip1}$ protein was not evident in HT-29 cells (Fig. 1B). Paclitaxel at a dose of 1 nM failed to induce nuclear p53 and $p21^{waf1/cip1}$ protein in both cell lines (Table 1). Significant nuclear induction of p53 and $p21^{waf1/cip1}$ proteins was observed in HCT-116 when radiation was combined with paclitaxel treatment, indicating that HCT-116 cells harbor functional p53. However, in HT-29 cells, combination treatment failed to induce these proteins, suggesting that these cells lack functional p53 protein (Table 1).

HT-29 Cells Show High Levels of Basal p53 Protein with the Absence of $p21^{waf1/cip1}$ by Western Blot Analysis

To ascertain the basal and radiation-induced total p53 protein levels and also its downstream target gene $p21^{waf1/cip1}$, Western blot analysis was performed after various time intervals using p53 and $p21^{waf1/cip1}$ antibodies. Similar to the results obtained by immunocytochemical analysis, HCT-116 cells showed an increase in p53 protein and its target gene $p21^{waf1/cip1}$ (Fig. 2A). However, in HT-29 cells, high p53 basal levels were detected and these levels remained unchanged after radiation. No basal level of $p21^{waf1/cip1}$ protein was detected in HT-29 cells and induction of this protein by radiation was not evident (Fig. 2B). Together, these data suggest that HT-29 cells lack wild-type p53 protein and HCT-116 cells contain func-

TABLE 1
Immunocytochemical Analysis of p53 and $p21^{waf1/cip1}$ Protein Expression in HCT-116 and HT-29 Cells Treated with Radiation or Paclitaxel or In Combination

Treatment	Cell lines	Time points	p53 expression	$p21^{waf1/cip1}$ expression
Radiation Alone (2 Gy)	HCT-116	UT	+	+
		6 hrs	+++	++
		24 hrs	++	+++
	HT-29	UT	++++	−
		6 hrs	++++	−
		24 hrs	++++	−
Paclitaxel alone (0.001 μM)	HCT-116	UT	+	+
		6 hrs	+	+
		24 hrs	+	+
	HT-29	UT	++++	−
		6 hrs	++++	−
		24 hrs	++++	−
Radiation (2 Gy) + paclitaxel (0.001 μM)	HCT-116	UT	+	+
		6 hrs	+++	++
		24 hrs	++	++
	HT-29	UT	++++	−
		6 hrs	++++	−
		24 hrs	++++	−

Gy: gray; UT: untreated; +; 1–25% of the cells were positive; +++: 51–75% of the cells were positive; ++: 25–50% of the cells were positive; ++++: 76–100% of the cells were positive; −: < 1% of the cells were positive.

tional p53, which may be responsible in up-regulating its downstream effector gene $p21^{waf1/cip1}$.

Radiation Caused Enhanced Clonogenic Inhibition in HCT-116 Cells

Radiation caused clonogenic inhibition in both cell lines. The estimates of radiation inactivation for the two cell lines are presented in Table 2. SF_2 for HCT-116 cells was found to be 0.383 with a D_0 value of 223 cGy (Fig. 3) (Table 2). However, SF_2 for HT-29 was 0.614 with a D_0 value of 351 cGy (Fig. 3) (Table 2). Thus, compared with the HT-29 cells, the HCT-116 cells were significantly more sensitive to ionizing radiation ($P < 0.007$). The paclitaxel alone D_0 values for HCT-116 and HT-29 were 0.7 nM and 1.11 nM, respectively (Fig. 4) (Table 2). Based on these findings, HCT-116 cells also were significantly more sensitive to paclitaxel than HT-29 cells ($P < 0.06$). The radiation plus paclitaxel (1 nM) D_0 values for HCT-116 and HT-29 were 103 cGy and 306 cGy, respectively (Fig. 5) (Table 2). The mean radiation ER for HCT-116 was found to be 2.5 and was 1.07 for HT-29. These data indicate that paclitaxel conferred a significantly greater radiation ER in HCT-116 wild-type p53 containing cells ($P < 0.006$) compared with mutant p53 HT-29 cells ($P < 0.31$).

Paclitaxel in Combination with Low Dose Multifractionated Radiation Conferred Radiosensitization in Mutant p53 HT-29 Cells

Because no significant radiosensitizing effect of paclitaxel was observed with a single dose of radiation in HT-29 cells harboring mutant p53, we further investigated whether fractionated radiation may alter the paclitaxel-mediated radiosensitization outcome. We designed experiments in which cells were treated with paclitaxel for 24 hours and irradiated with either 2 1-Gy dose fractions or 4 0.5-Gy dose fractions separately with an 8-hour interval between each fraction. HCT-116 cells showed an ER of 2.17, 2.5, and 2.29 for the 2-Gy dose alone, 2 1-Gy fractions, and 4 0.5-Gy fractions, respectively. These results suggest that radiosensitization by paclitaxel in HCT-116 cells was achieved regardless of single or multifractionated radiation exposure (Fig. 6). However, HT-29 cells showed an ER of 1.05, 1.46, and 2.18 for the 2-Gy dose alone, 2 1-Gy fractions, and 4 0.5-Gy fractions, respectively (Fig. 7). This observation suggests that failure of radiosensitization with a single 2-Gy radiation dose may be due to the influence of mutant p53 status. However, radiosensitization increased significantly when low dose radiation was delivered as two 1-Gy fractions ($P < 0.02$) or in 4 0.5-Gy fractions ($P < 0.001$). These results indicate that low dose fractionated radiation in combination with paclitaxel may overcome the influence of the radioresistant phenotype caused by mutant p53.

DISCUSSION

Mutations and deletions of p53 have been identified in approximately 50% of colorectal carcinomas.[24] The p53 gene is an essential component of the pathway leading to apoptosis caused by DNA damage.[4] Wild-type p53 protein confers radiation responsiveness, which causes either G_1 cell cycle arrest and/or apoptotic death resulting from activation of other downstream target genes such as $p21^{waf1/cip1}$, GADD45, and MDM2.[3,4] Induction of nuclear $p21^{waf1/cip1}$ protein leads to inhibition of the cyclin-dependent kinase complex,[25,26] which results in accumulation of the unphosphorylated retinoblastoma (Rb) gene product.[27] Hypophosphorylated Rb abrogates the activation of the E2F transcription factors that otherwise would signal entry into S-phase.[28] Together, these mechanisms lead to G_1 arrest, which allows the cell to repair DNA damage.[29] In the current study, radiation caused an increase in nuclear p53 and $p21^{waf1/cip1}$ proteins in HCT-116 cells, suggesting that nuclear induction of p53 involved the induction of its downstream effector gene $p21^{waf1/cip1}$. This is supported by the fact that

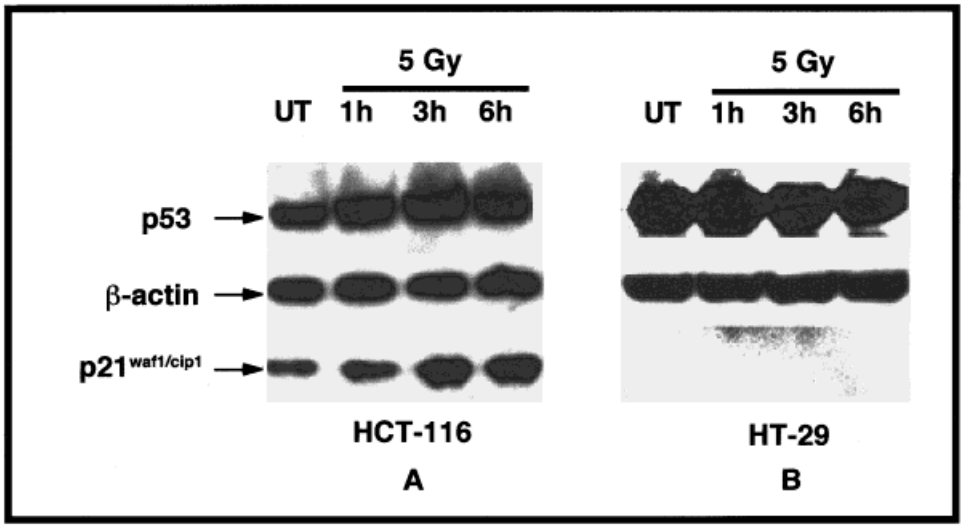

FIGURE 2. Western blot analysis of p53 and $p21^{waf1/cip1}$ induction in HCT-116 and HT-29 cells. Cells were left untreated or were irradiated and subjected to Western blot using anti-p53 antibody (D0-1) or anti-p21 antibody or β-actin for internal loading controls. Gy: gray; h: hour(s).

TABLE 2
Estimates of Radiation Inactivation in HCT-116 and HT-29 Cells Using Single Dose Radiation Alone, Paclitaxel Alone, and In Combination

	HCT-116					HT-29				
			ER					ER		
Treatment	SF_2	D_0	SF_2 ER	D_0 ER	Mean of SF_2 ER + D_0 ER	SF_2	D_0	SF_2 ER	D_0 ER	Mean of SF_2 ER + D_0 ER
Radiation alone	0.383	223 cGy	–	–	–	0.614	351 cGy	–	–	–
Paclitaxel alone	–	0.7 nM	–	–	–	–	1.11 nM	–	–	–
Radiation + paclitaxel	0.138	103 cGy	2.77	2.16	2.5	0.608	306 cGy	1	1.15	1.075

ER: enhancement ratio; SF_2: survival fraction of exponentially growing cells that were irradiated at the clinically relevant dose of 2 grays; D_0: dose required to reduce the fraction of cells to 37%, which is indicative of a single event killing; cGy: centigray.

these cells contain wild-type p53 with no obvious mutation at the DNA level.[21] Early reports also have shown that in HCT-116 cells, ionizing radiation caused up-regulation of p53 and $p21^{waf1/cip1}$ protein.[21] However, in HT-29 cells, no induction of nuclear p53 and $p21^{waf1/cip1}$ protein was observed after radiation treatment. Ho et al. have shown that when HT-29 cells were treated with nitric oxide, no elevation of p53 protein was observed.[30] Thus, the absence of induction in these genes may be due to the presence of a mutated p53 gene in HT-29 cells, which previously have been shown to contain a point mutation (Arg-His) at codon 273.[22]

In certain cell types, the loss of p53 function causes enhanced resistance to ionizing radiation.[31] In head and neck squamous cell carcinoma cell lines, to our knowledge, no correlation between p53 function and radiosensitivity has been demonstrated to date.[32,33] The correlation between p53 function and radiosensitivity may be a tissue specific phenomenon.[29] By clonogenic assay, the results of the current study demonstrated that the wild-type p53 containing HCT-116 cells was sensitive to radiation compared with HT-29 cells harboring mutant p53. Stromberg et al also showed that HT-29 cells were highly radioresistant compared with the most radiosensitive breast carcinoma cell line MCF-7, which contains the wild-type p53 gene.[34] Sensitivity to radiation by HCT-116 cells may be due to the presence of nuclear induction of p53 and $p21^{waf1/cip1}$ proteins, which previously was found to be associated with enhanced radiation response.[35]

Numerous studies have shown a correlation between p53 status and paclitaxel sensitivity. Recently, Rakovitch et al. reported that RKO colorectal carcinoma cells lacking functional p53 showed enhanced

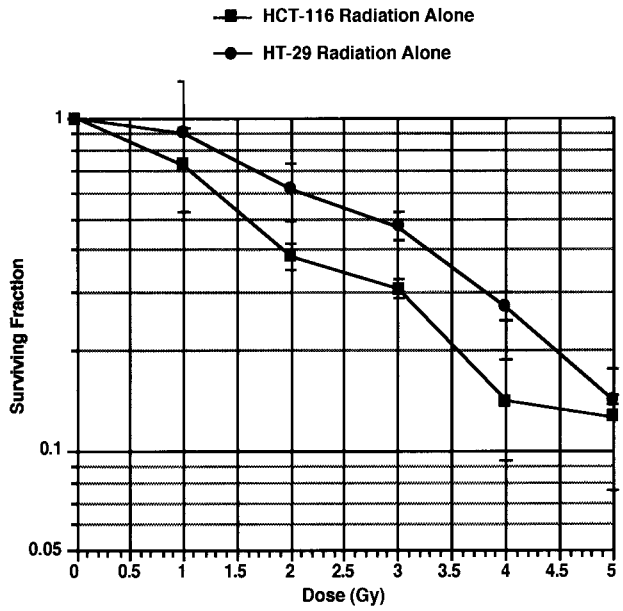

FIGURE 3. Radiation-induced clonogenic inhibition in HCT-116 and HT-29 cells. Gy: gray.

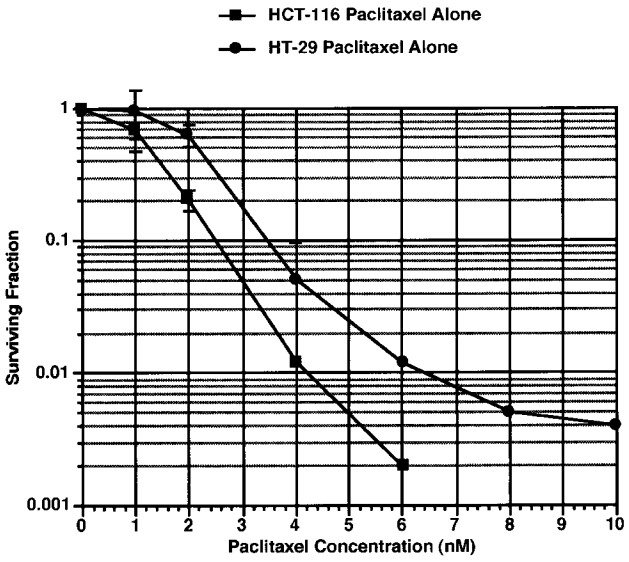

FIGURE 4. Paclitaxel-induced clonogenic inhibition in HCT-116 and HT-29 cells.

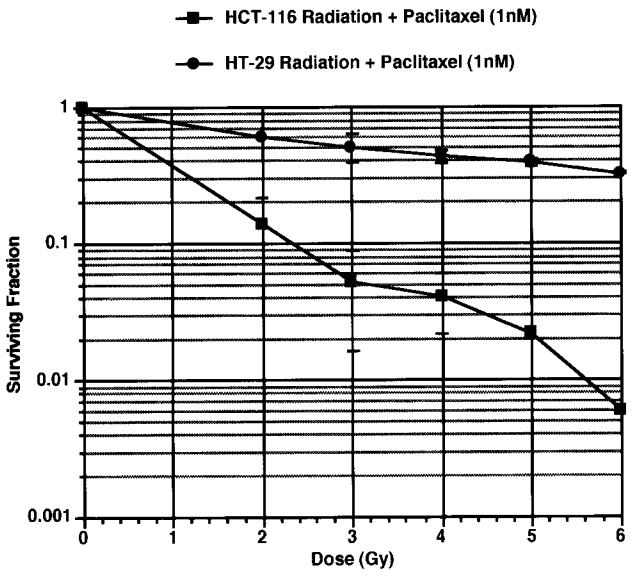

FIGURE 5. Radiation plus paclitaxel-induced clonogenic inhibition in HCT-116 and HT-29 cells. Gy: gray.

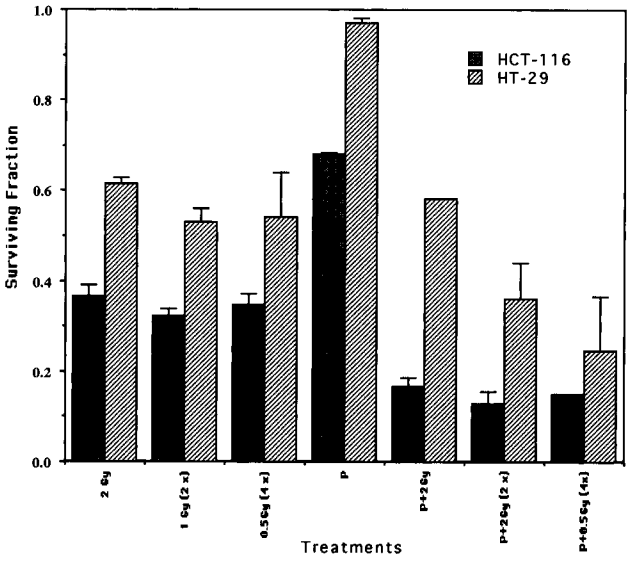

FIGURE 6. Estimates of surviving fraction by 2 gray (Gy) fractionated radiation exposure alone or in combination with paclitaxel. P: paclitaxel; 2×: 1-gray fraction 2 times; 4×: 0.5-Gy fraction 4 times.

sensitivity to paclitaxel compared with wild-type p53 RKO cells.[36] In contrast, it was shown that mutant p53 did not predict a response to paclitaxel in nonsmall cell lung carcinoma and paclitaxel was found to bypass mutant p53, thereby leading to tumor cell death by an alternative pathway.[37] The disruption of p53 in lymphoid, breast, and colon carcinoma cell lines also do not appear to affect paclitaxel sensitivity.[22] The increased sensitivity to paclitaxel was not associated with p53 abrogation. However, this has not been observed consistently.[10] In the current study, paclitaxel caused greater clonogenic inhibition in HCT-116 cells than in HT-29 cells. Simultaneously, we also found that paclitaxel failed to induce nuclear p53 and $p21^{waf1/cip1}$ proteins in both HCT-116 and HT-29 cells. Therefore, the increased sensitivity in HCT-116 cells may be due to the induction of alternate pathways for clonogenic inhibition by paclitaxel that may be independent of wild-type p53.

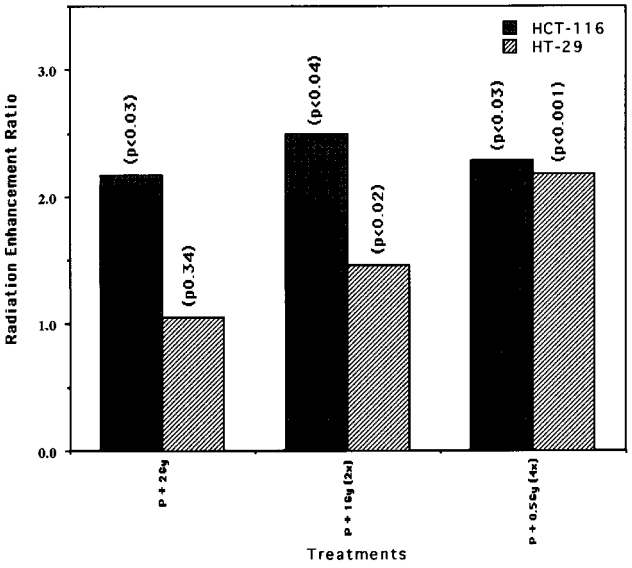

FIGURE 7. Radiation enhancement ratios in cells treated with a combination of fractionated radiation and paclitaxel. P: paclitaxel; 2×: 1-Gy fraction 2 times; 4×: 0.5-Gy fraction 4 times.

Arrest in the G_2/M-phase of the cell cycle is a major mechanism of action by paclitaxel.[38] Because enhanced radiation sensitivity occurs most often when radiation is delivered at the point of cell accumulation in the G_2/M-phase, paclitaxel was found to be a potent radiosensitizer.[16] There is ample evidence from the literature that paclitaxel can enhance radiosensitivity in many tumor cell lines with radiation enhancement ratios ranging from 1.1 to > 3.0.[16] Even additive[34,39–41] and subadditive effects have been reported.[41–43] The variation in radiation enhancement ratios may be due to cell type, proliferation state, drug concentration, and timing of radiation delivery in relation to drug administration. In the current study, the focus primarily was on the influence of p53 status on the radiosensitization potential of paclitaxel. HCT-116 cells showed a supradditive effect with a mean radiation ER of 2.5. However, in HT-29 cells, no radiosensitization effect was observed at 1 nM (mean ER of 1.0). Stromberg et al. found similar results in which paclitaxel failed to produce a radiosensitizing effect in HT-29 cells, even at concentrations of 5 or 10 nM.[34] These observations may suggest that intact p53 gene function may be a necessary component to regulate the radiosensitization effect of paclitaxel.

To overcome the negative impact of mutant p53 on paclitaxel radiosensitization, other mechanisms for the enhancement of cell killing may be necessary. HT-29 cells do not exhibit a G_2/M-phase block when exposed to paclitaxel; however, radiation was found to induce a strong G_2/M-phase block in these cells.[34] Because the effect of paclitaxel is pronounced in the G_2/M-phase, we hypothesized that multiple fractionated radiation may cause a reverse sensitization of cells to paclitaxel (i.e., radiation acting as a chemosensitizer). To test this hypothesis, cells were exposed to 1 nM of paclitaxel for 24 hours and then irradiated to 0.5-Gy or 1-Gy fractions to a total dose of 2 Gy with an 8-hour delay between each fraction. HCT-116 cells showed a radiation ER of 2.5 for a 1-Gy fraction or 2.3 for a 0.5-Gy fraction; this ratio was similar to that for a single radiation dose. It is interesting to note that in HT-29, superadditive action was evident in both the fractions. The increase in the radiation ER was directly proportional to the number of fractions used. With 2 fractions of 1 Gy, the ER was 1.49 and was 2.18 with 4 fractions of 0.5 Gy (Fig. 7). Thus, this finding strongly indicates that to overcome the negative impact of mutant p53, low dose fractionated radiation therapy with pretreatment with paclitaxel can enhance cell death and that this may be due to chemosensitization of the cells by radiation. Van Rijn et al. reported that an additional inhibition of cell proliferation in human lung carcinoma cells was observed at low concentrations of paclitaxel when combined with fractionated radiation.[44] It also was found that enhancement of the sensitization effect by the combination of paclitaxel and radiation was caused by increasing the α component of the cell survival curves. These results suggest that there is likely to be minimal benefit from the addition of paclitaxel to radiation in the treatment of tumors with mutant p53. However, fractionated low dose radiation (multiple small fractions per day) may render enhanced chemosensitization, resulting in tumor control.

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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-potentiation 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-potentiated 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 SCCHN[2] 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 G_1 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 G_2 and M phases of the cell cycle (12, 13). This ability of Paclitaxel to arrest cells in G_2-M makes it a potential radiosensitizer. Thus, G_2-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; NFκB, nuclear factor κ B; ER, enhancement ratio; TUNEL, terminal transferase-mediated dUTP-digoxigenin nick end labeling; EMSA, electrophoretic mobility shift assay; SF, surviving fraction; D_0, 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-potentiation 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% CO_2.

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 multifractionated 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:

Radiation ER = [survival fraction of radiation[3] alone]/[survival fraction of radiation[3] + Paclitaxel[4]]

Paclitaxel ER by radiation was calculated using the following formula:

Paclitaxel ER = [survival fraction of Paclitaxel alone]/[survival fraction of radiation[3] + 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:

Radiation ER = [percentage of induction of apoptosis by radiation[5] + Paclitaxel[6]]/[percentage of induction of apoptosis by radiation[5]]

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

Paclitaxel ER = [percentage of induction of apoptosis by radiation[5] + Paclitaxel[6]]/[percentage of induction of apoptosis by Paclitaxel[6]]

Definition of the terms "Radio-Sensitization" and "Chemo-Potentiation." Terms such as radio-sensitization and chemo-potentiation 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.

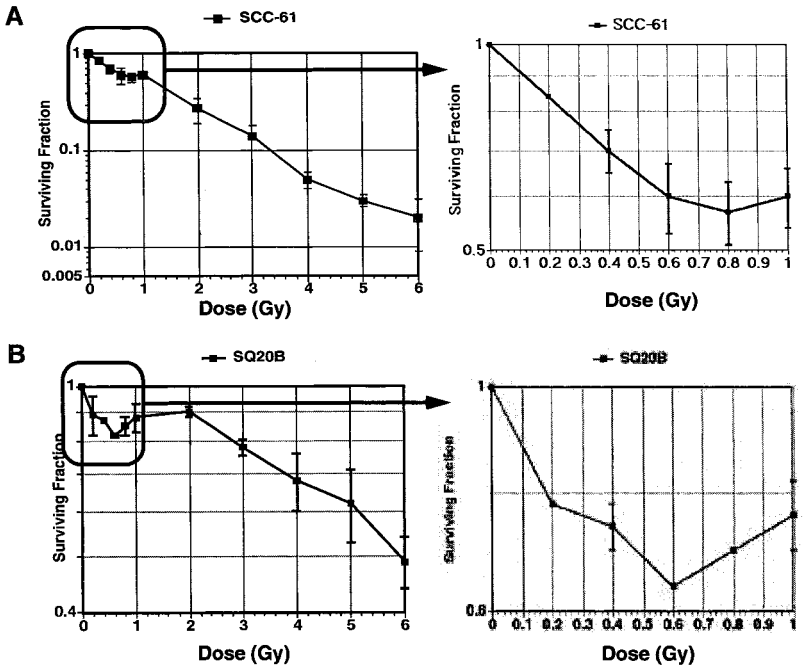

Fig. 1 Presence of HRS phenomenon at low-dose radiation in SCC-61 cells (*A*) and SQ-20B cells (*B*). Cell survival curves for SCC-61 and SQ-20B cells after radiation as analyzed by Single Hit Multi-Target model curve fit. Error bars, SE from the mean of three separate experiments.

Chemo-potentiation 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 ^{32}P-labeled, double-stranded oligonucleotide probe with a high affinity for NFκB binding (Promega, Madison, WI). Binding reactions were electrophoresed on a 4% PAGE in 0.5 × 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 (SF_2 and D_0) 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 SQ20B 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 D_0 values for SCC-61 and SQ-20B were

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

	SCC-61				SQ-20B			
			Enhancement ratios				Enhancement ratios	
			Radio-sensitization[a]	Chemo-potentiation[b]			Radio-sensitization[a]	Chemo-potentiation[b]
Treatments	SF[c]	D_0	SF ER	SF ER	SF	D_0	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; D_0, 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-potentiation 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.

LDFRT 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. 2*A*). In combination with Paclitaxel, two fractions of 1 Gy reduced the SF significantly ($P < 0.0034$) in both cell lines (Fig. 2*A*). 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. 2*A*). 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. 2*B*). 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. 2*B*). 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. 2*B*). 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-potentiation, 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. 2*C*). 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. 2*C*). 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. 2*C*). Thus, these findings strongly indicate that LDFRT is a potent chemo-potentiator of Paclitaxel as opposed to single radiation dose-mediated chemo-potentiation.

LDFRT 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. 3*A*). A proportionate increase in cell death was observed in both cell lines when Paclitaxel and single 2 Gy radiation dose were combined (Fig. 3*A*). Thus, in terms of apoptotic radiosensitization by Paclitaxel, the radiation ER for cell death was 1.64 and 1.77 in SCC-61 and

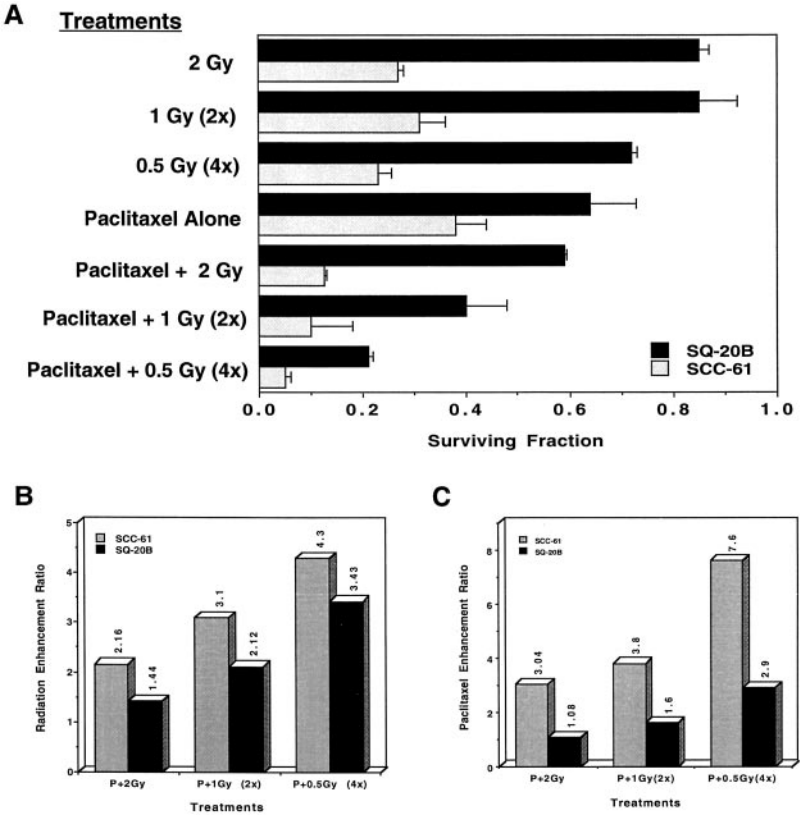

Fig. 2 Bar graph showing SFs for SCC-61 and SQ-20B cells treated with Paclitaxel (0.5 nM) plus single 2 Gy of radiation dose and Paclitaxel (0.5 nM) plus LDFRT, respectively (*A*). Bar graph showing radiation ERs for SCC-61 and SQ-20B cells treated with Paclitaxel (0.5 nM) plus single 2 Gy of radiation 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. 3*B*). These findings indicate that Paclitaxel conferred significant radio-sensitizing apoptotic effects, irrespective of p53 status. In terms of apoptotic chemo-potentiation by single 2 Gy radiation dose, the Paclitaxel ER was 3.1 and 4.86 for SCC-61 and SQ-20B cells, respectively (Fig. 3*C*). 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. 3*B*). 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. 3*C*). 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 NFκB 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 NFκB, 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. 4*A*). LDFRT in combination with Paclitaxel caused significant induction of BAX with significant down-regulation of BCL-2 (Fig. 4*A*). NFκB, 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 NFκB activity in SCC-61 cells (Fig. 4*B*). Paclitaxel alone and Paclitaxel in combination with LDFRT did not show any presence of NFκB activity (Fig. 4*B*). 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. 5*A*). Induction of NFκB activity was observed by single 2 Gy radiation dose but not with LDFRT. Paclitaxel alone caused a marginal induction of NFκB activity with no significant changes in BCL-2 or BAX protein (Fig. 5*B*). When Paclitaxel was combined with LDFRT, significant induction of BAX was observed in all 0.5 Gy fractions (Fig. 5*A*). A weak induction of NFκB 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 antiapoptotic factors, such as BCL-2 and NFκB.

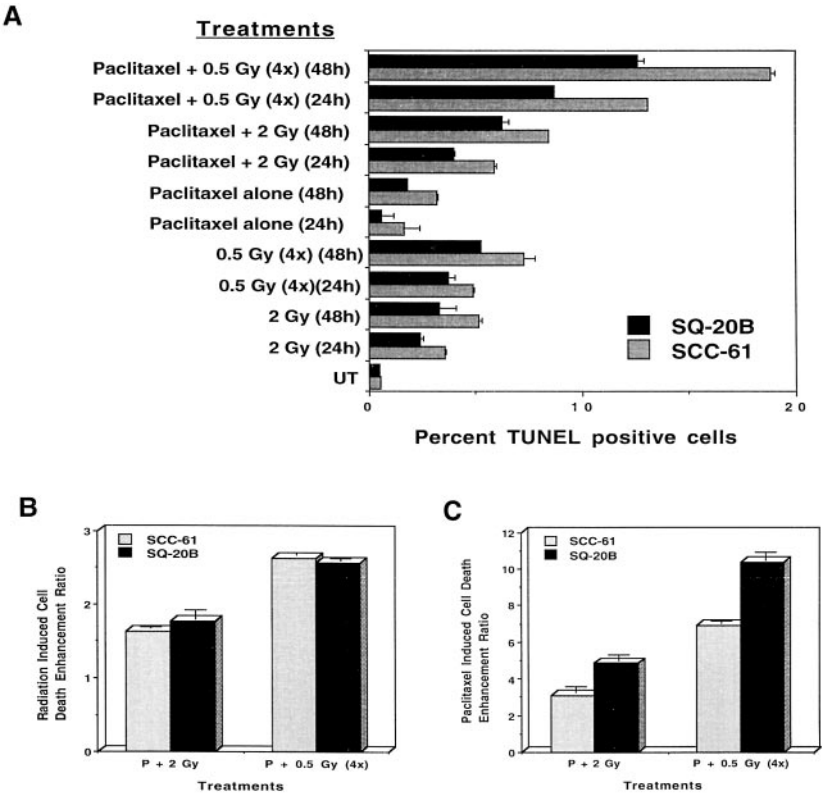

Fig. 3 Bar graph showing radiation-induced cell death (as a percentage of TUNEL-positive cells) for SCC-61 and SQ-20B cells treated with Paclitaxel (0.5 nM) plus single 2 Gy of radiation dose and Paclitaxel (0.5 nM) plus LDFRT, respectively (*A*). Bar graph showing radiation-induced cell death ERs for 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 G_1 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 G_1 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 G_2-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-

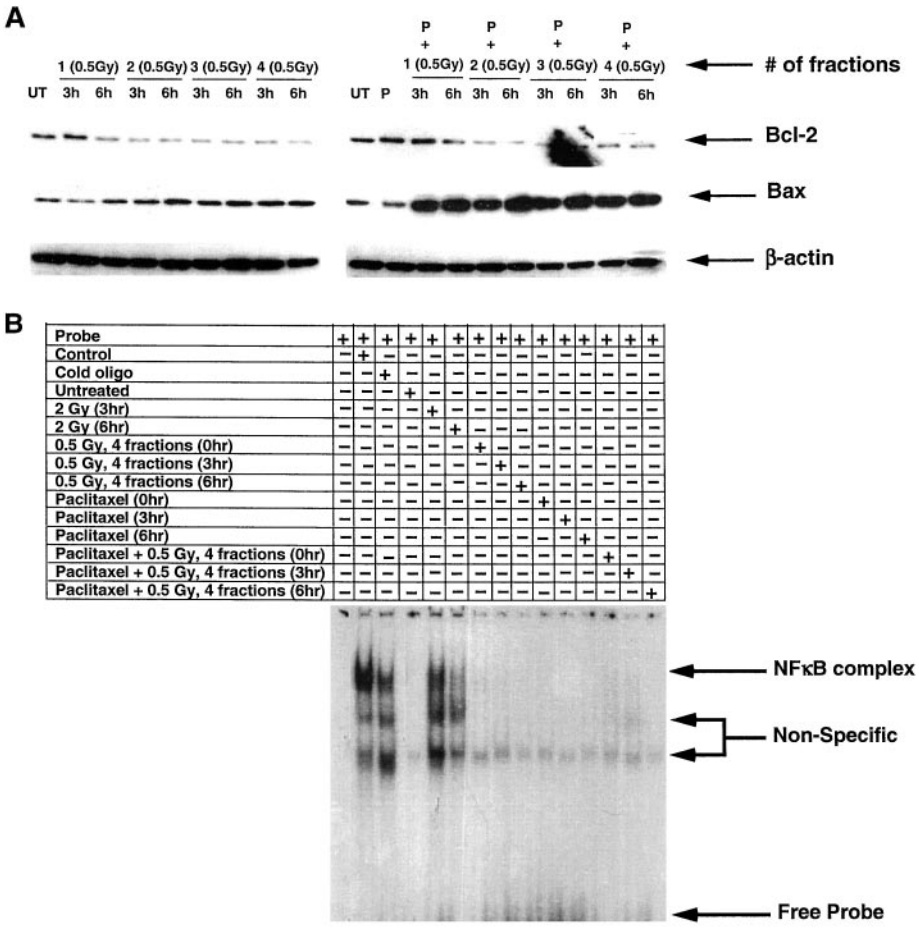

Fig. 4 Effect of LDFRT or LDFRT plus Paclitaxel on kinetics of pro-apoptotic and pro-survival factors in SCC-61 cells. *A,* Western blot analysis showing BCL-2 and BAX kinetics in SCC-61 cells. The cells were exposed to LDFRT alone or in combination with Paclitaxel plus LDFRT. The lysates isolated from Paclitaxel alone group are 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 SCC-61 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 ^{32}P-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.

comes the BCL-2-mediated radiation resistance in p53 mutant SQ-20B. Human cells [adenocarcinoma cells of human breast (MCF-7), lung (A-549), ovary (OVG-1), and pancreas (PC-Sh)] exposed to Paclitaxel for 24 h at concentrations ranging from 2.5 to 50 nM showed a sharp decline in SF (40). In this study, we observed similar results using much lower doses of Paclitaxel, and this may be attributable to maintaining Paclitaxel drug concentration in the medium throughout the experiment.

This study demonstrated that Paclitaxel caused enhanced radio-sensitization, irrespective of p53 status; however, chemo-potentiating effects of single 2 Gy dose radiation on Paclitaxel were not observed in p53 mutant SQ-20B cells. Thus, in the SCCHN background, we used a novel concept of LDFRT not only to achieve greater radio-sensitization effects of Paclitaxel but also enhance chemo-potentiating effects of radiation (LDFRT), irrespective of p53 status. Low doses of radiation (10–60 cGy) were found to induce HRS phenomenon, and doses $>$ 1 Gy demonstrated IRR (15). Low doses of 0.5 Gy in fractionated form significantly potentiated the effects of Paclitaxel and caused enhanced radiosensitization in both cell lines, irrespective of p53 function. It is clear from the previous reports that radiation at higher doses ($>$100 cGy) leads to IRR, and this is corroborated by our molecular analysis whereby cells exposed to 2 Gy radiation dose caused an increase in NFκB activity. NFκB activity often targets the induction of BCL-2 protein and thereby produces radio-resistance among tumor cells (26). This molecular signaling may be the basis of IRR. On the other hand, low doses of radiation that induce HRS phenomenon were found to cause a significant increase in the pro-apoptotic protein BAX, with no induction of NFκB activity, suggesting that the low doses of radiation have the potency to selectively induce pro-apoptotic pathways by inhibiting pro-survival pathways and thus eliminating the quandary of IRR.

These data support strong consideration for clinical trials using LDFRT as a chemo-potentiator in head and neck cancer to overcome the chemo- and radio-resistance. A recently completed pilot trial using LDFRT in combination with Carboplatin/Taxol as induction therapy was done in patients with locally advanced head and neck cancer.[7] This novel approach provided a response rate of 89% at the primary site, 71% at neck nodes,

[7] S. M. Arnold, W. F. Regine, J. Valentino, P. Spring, P. Desimone, M. Kudrimoti, D. Kenady, M. M. Ahmed, C. Lee, and M. Mohiuddin. Low-dose fractionated radiation (LDFRT) as a chemoenhancer of neo-adjuvant Paclitaxel (P) and Carboplatin (CBCDA) for locally advanced squamous cell carcinoma of the head and neck (SCCHN): results of a new treatment paradigm, manuscript in preparation.

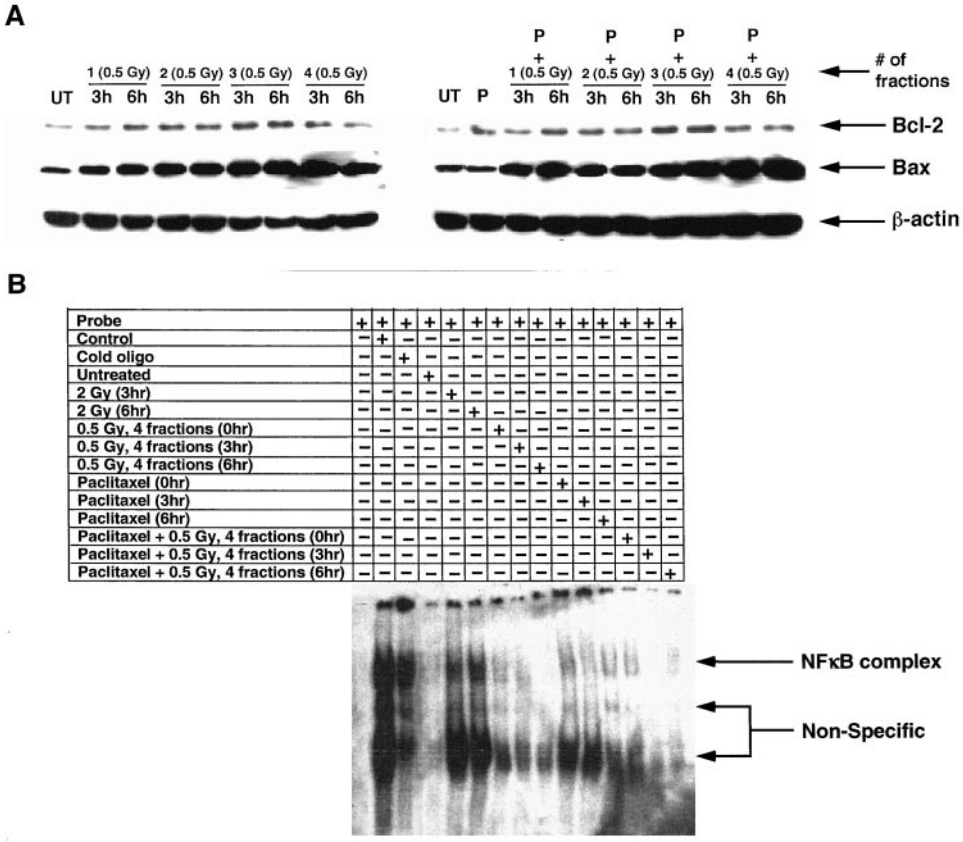

Fig. 5 Effect of LDFRT or LDFRT plus Paclitaxel on kinetics of pro-apoptotic and pro-survival factors in SQ-20B cells. *A,* Western blot analysis showing BCL-2 and BAX kinetics in SQ-20B cells. The cells were exposed to LDFRT alone or in combination with Paclitaxel plus LDFRT. The lysates isolated from 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 ^{32}P-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-potentiation and also eliminate IRR.

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CLINICAL INVESTIGATION **Head and Neck**

LOW-DOSE FRACTIONATED RADIATION AS A CHEMOPOTENTIATOR OF NEOADJUVANT PACLITAXEL AND CARBOPLATIN FOR LOCALLY ADVANCED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK: RESULTS OF A NEW TREATMENT PARADIGM

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Purpose: Current therapies for locally advanced squamous cell carcinoma of the head and neck (SCCHN) result in 50% long-term remission. Low-dose radiotherapy (<100 cGy) induces enhanced cell killing *in vitro* via the hyper-radiation sensitivity phenomenon but has not been used in the clinical setting. On the basis of the demonstrated synergy between chemotherapy and low-dose fractionated RT, a novel neoadjuvant therapy was designed using low-dose fractionated RT as a chemopotentiator for locally advanced SCCHN.

Methods and Materials: Forty patients with locally advanced SCCHN received paclitaxel (225 mg/m^2), carboplatin (area under the curve of 6), and four 80-cGy fractions of radiotherapy (two each on Days 1 and 2). This sequence was repeated on Days 22 and 23.

Results: Of the 40 patients enrolled, 39 were assessable. Grade 3 or worse toxicities included neutropenia (50%), infection (13%), arthralgias/myalgias (3%), skin (8%), lung (3%), and allergic reaction (3%), with no Grade 5 toxicity. The response was assessed radiographically and by panendoscopy. At the primary site, 11 patients (28%) had a complete response, 24 (62%) had a partial response, and 4 (10%) had stable disease. Of those with lymph node involvement, 10 (31%) had a complete response, 12 (38%) a partial response, 9 (28%) had stable disease, and 1 (3%) had progressive disease. The overall response rate was 82%.

Conclusion: Low-dose fractionated RT combined with paclitaxel and carboplatin is effective in SCCHN and has a similar toxicity profile to chemotherapy alone. This novel approach provided a response rate of 90% at the primary site and a nodal response rate of 69%. Additional scientific investigation of this new treatment paradigm is warranted. **© 2004 Elsevier Inc.**

Low-dose, Radiotherapy, Induction, Chemotherapy.

INTRODUCTION

More than 40,000 new cases of squamous cell cancer of the head and neck (SCCHN) are diagnosed annually. Surgery followed by radiotherapy (RT) or RT alone provides a 5-year survival rate of 30–40% (1). This poor survival has spurred investigation into more aggressive concurrent chemotherapy and RT strategies, supported by excellent preclinical evidence of synergy between RT and chemotherapy (2–4). Although the most effective schedule and combination of chemotherapeutic agents with RT remains controversial, concurrent chemoradiotherapy offers an improved 3–5-year survival rate of 50% (5–8). Emerging data from aggressive concurrent chemoradiotherapy have revealed a paradigm shift in the patterns of failure in SCCHN, with the distant metastatic rate exceeding the locoregional failure rate (9–11). The improvement in locoregional control rates and increasing percentage of distant metastatic disease has led to a reexamination of the importance of systemic therapy in SCCHN.

Neoadjuvant combination chemotherapy has been used to address this increasing distant metastatic rate, with the added hope of improved survival in advanced SCCHN. Despite encouraging response rates (RRs) from 60% to 90%, the benefit of induction therapy on survival and con-

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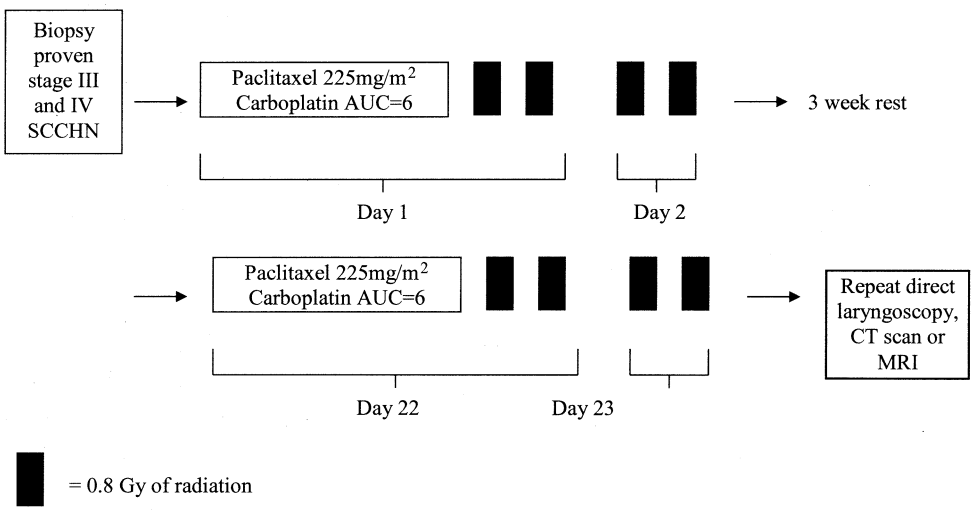

Fig. 1. Regimen for low-dose fractionated radiotherapy, carboplatin, and paclitaxel. Solid bars represent 80-cGy fractions of RT.

trol of metastatic disease has been intensely debated in the literature (12–16). However, induction chemotherapy has established benefit in organ preservation and in predicting the response to subsequent, definitive RT and chemotherapy (15, 17–19). Early studies evaluated cisplatin–5-fluorouracil combinations (13, 16–18), but many investigators have moved to taxane–platinum combinations (15, 20–24) because of improved efficacy *in vitro* and in other tumor sites (i.e., lung cancer). Taxane–platinum combinations are especially beneficial in head-and-neck cancer for several reasons—they provide a range of RRs from 55% to 87% (15, 22–24) in SCCHN, have complementary mechanisms of action, are excellent radiation sensitizers, and have a low rate of mucositis and safe overall toxicity profile when given concurrently with RT.

Joiner and colleagues (25) revolutionized thinking about low doses of RT (<100 cGy) by demonstrating an initial phase of hyper-radiosensitivity (HRS) using doses from 0 to 80 cGy. The HRS observed with low-dose RT is a unique radiobiologic phenomenon (26). We recently reported that low-dose RT delivered in fractionated form (ultrafractionation) acts synergistically with chemotherapy *in vitro* (27). Preclinical data have indicated that the maximal HRS phenomenon occurs at a dose between 50 and 80 cGy and that four low-dose fractions provide optimal cell killing *in vitro* when combined with chemotherapy (27). On the basis of these findings, we designed a novel induction therapy of paclitaxel, carboplatin, and low-dose fractionated RT (LDFRT) in advanced SCCHN before definitive surgery or RT to exploit this unique synergy (28). This protocol allowed evaluation of two important endpoints: the toxicity of LDFRT plus carboplatin and paclitaxel and the clinical response to therapy. This report describes the Phase II results of this new treatment paradigm.

METHODS AND MATERIALS

Patient characteristics and eligibility criteria

Between July 2000 and May 2002, 40 patients with locally advanced primary SCCHN were treated at the University of Kentucky on a Phase II protocol (00-H&N-11) using LDFRT, paclitaxel, and carboplatin. All patients signed an informed consent form approved by our institutional review board and according to federal guidelines. Patients were required to have pathologically documented Stage III or IV SCCHN (excluding M1 disease) within 2 months of diagnosis. Before enrollment, all patients underwent CT or MRI of the involved area of the head and neck, chest X-ray or chest CT scan, and direct laryngoscopy with biopsy of the affected area. Patients were required to have an Eastern Cooperative Oncology Group performance status of ≥2, no evidence of active cardiac abnormalities, adequate bone marrow reserve (absolute neutrophil count of >1000/μL, platelet count >100,000/μL), serum total bilirubin ≤1.5 mg/dL, and a calculated or measured creatinine clearance >60 mL/min. Patients were excluded if they had a history of malignancy within the past 5 years (other than nonmelanomatous skin cancer or carcinoma *in situ* of the cervix) or preexisting peripheral neuropathy greater than Grade 1.

Treatment and evaluation

Induction chemotherapy and RT. The treatment scheme for this study is shown in Fig. 1. All chemotherapy was calculated using the patient's actual body weight and administered in the outpatient chemotherapy infusion center. Paclitaxel was diluted in 0.9% sodium chloride to a final concentration of 0.3–1.2 mg/mL and was given at a dose of 225 mg/m^2 i.v. within 3 h on Days 1 and 22. After the paclitaxel infusion on Days 1 and 22, carboplatin, reconstituted in 0.9% sodium chloride to a final concentration of

approximately 10 mg/mL, was given within 30 min at an area under the curve of 6, calculated using the Calvert formula. To avoid the allergic reactions associated with paclitaxel, dexamethasone 20 mg, cimetidine 300 mg, and diphenhydramine 25 mg were given 30 min before each dose. The use of other premedications and antiemetics was left to the discretion of the treating physician.

Radiotherapy was given on Days 1 and 2 using four doses of 80 cGy each. The first fraction was given within 2 h of the completion of chemotherapy and the second fraction was given 4–6 h later. The third and fourth fractions were given on Day 2, 4–6 h apart (Fig. 1). RT was repeated as above on Days 22 and 23 of therapy. The patient was treated with shaped fields encompassing gross disease only (including the primary tumor and gross nodal disease) with a maximal 2-cm margin. The spinal cord was excluded from the radiation field, and CT-based treatment planning was used as needed and appropriate (in most patients). The total radiation dose for induction therapy was 640 cGy. A Phase I trial was not pursued because the safety data regarding RT and chemotherapy already existed for much greater doses of RT, and it was assumed that low-dose RT would be tolerated.

Posttherapy evaluation. Radiographic tumor assessment by panendoscopy and CT or MRI was performed 3 weeks after the second cycle of chemotherapy and RT. The attending otolaryngologist assessed the primary site response at the time of panendoscopy. A complete response (CR) was defined as the complete disappearance of all measurable disease; a partial response (PR) as a >50% reduction in the sum of the product of the perpendicular diameters of the prospectively identified index lesions (before treatment on protocol), with no progression in any lesion; stable disease (SD) as less than a PR, but without progression in any lesion; and progressive disease (PD) as an increase in any measurable lesion or the appearance of any new lesion. The nodal response was assessed clinically and radiographically and was scored separately from the primary tumor response, using the same definitions and more than one nodal group whenever possible. The overall response was graded on the basis of the primary and nodal response, as above. Toxicity evaluations were performed before the study and after the second cycle of therapy. Toxicities were scored using the National Cancer Institute Common Toxicity Criteria (29).

Statistical analysis

The primary objective of this Phase II study was to evaluate the antitumor response rate and toxicity of the combination of carboplatin, paclitaxel, and chemopotentiating RT in locally advanced head-and-neck cancers. A two-stage Phase II trial design was planned using the study design by Simon (30). The treatment would be considered worthy of further investigation if no Grade 5 toxicity was seen and a RR of ≥50% was seen on the basis of the published data available at the time of study design (24). With an α level of 0.05 and a β level of 0.20, this study had an 80% probability of detecting a statistically significant difference between the RR for the proposed regimen and the RR for induction chemotherapy in historical controls (22, 24). Initially, 23 patients were enrolled in the study. At the time of interim analysis, >13 of the 23 patients had demonstrated an objective response to the proposed therapy, so the study was continued to a final target accrual of 40 patients.

Table 1. Patient characteristics

Characteristic	*n*	%
Gender		
Male	32	80
Female	8	20
Age (y)		
Range	36–81	
Median	56	
Location		
Oropharynx	15	37.5
Larynx	13	32.5
Oral cavity	6	15
Hypopharynx	5	12.5
Maxillary sinus	1	2.5
Total	40	100

RESULTS

Patients

Forty patients with locally advanced SCCHN were enrolled in this study. The median follow-up was 18 months (range, 7–29 months). Patient demographics are displayed in Table 1. Nine patients (22.5%) had Stage III disease, 31 (77.5%) had Stage IV disease, 18 (45%) had T4 disease, and 24 (60%) had advanced neck disease (Table 2). All patients were chemotherapy naïve, and in all but 1 patient this was the first diagnosis of SCCHN.

Thirty-nine patients were assessed for response and toxicity; 1 was lost to follow-up after the first cycle of chemoradiotherapy and was not evaluated. Patients were analyzed on an intent-to-treat basis. Two patients received only one of the planned two cycles of chemotherapy. One patient refused additional chemoradiotherapy after his first cycle, but was analyzed with the treatment group. One patient showed disease regression and symptomatic improvement at the primary site, but progression in the neck lymph nodes after the first cycle of therapy and was removed from study

Table 2. Tumor and nodal stage

	T1	T2	T3	T4	Total
N0			4	4	8
N1			5	3	8
N2a					0
N2b	1	3	3	5	12
N2c		3	2	4	9
N3			1	2	3
Total	1	6	15	18	40

Table 3. Grade 1–4 acute toxicity

Toxicity	NCI toxicity grade 1	2	3	4
Neutropenia	3 (8)	2 (5)	5 (13)	15 (38)
Anemia	8 (21)	4 (10)		
Thrombocytopenia	1 (3)	2 (5)		
Infection/Fever	1 (3)	7 (18)	4 (10)	1 (3)
Neutropenic			2 (5)	1 (3)
Nonneutropenic	1 (3)	7 (18)	2 (5)	
Arthralgias/myalgias	4 (10)	16 (41)	1 (3)	
Nausea	7 (18)	10 (26)		
Alopecia	7 (18)	21 (54)		
GI*		4 (10)		
Mucositis	1 (3)	4 (10)		
Allergic			1 (3)	
Neuromotor	1 (3)	2 (5)		
Fatigue	1 (3)	3 (8)		
Dysuria	2 (5)	2 (5)		
Dermatologic			3 (8)	
Pulmonary		1 (3)	1 (3)	
Dehydration		1 (3)		
Elevated ALT		1 (3)		
Weight loss	1 (3)	1 (3)		

Abbreviations: NCI = National Cancer Institute; ALT = alanine triphosphate.
Data presented as the number of patients, with the percentage in parentheses.
* Diarrhea, constipation, abdominal pain.

but included in the toxicity and response analysis. All remaining patients received the full intended dose of chemotherapy; only 1 patient had a 1-week delay in the delivery of the second cycle of chemotherapy.

Acute toxicity

Grade 3 and 4 toxicities included neutropenia (50%), infection (8%), dermatologic reactions (8%), allergic reactions (3%), pulmonary reactions (3%), and arthralgias/myalgias (3%). No Grade 5 toxicity occurred during therapy. All toxicities are outlined in Table 3.

Primary and nodal response

The responses are summarized in Table 4. Of the 39 analyzed patients, 11 (28%) had a CR at the primary site, 24 (62%) had a PR, and 4 (10%) had SD, for a primary site RR of 90%. Of the 32 analyzed patients with lymph node involvement, 10 (31%) had a CR, 12 (38%) had a PR, 9 (28%) had SD, and 1 (3%) had PD, for a nodal RR of 69%. In the 39 patients, the overall response rate was 82%; 5 (13%) had an overall CR and 27 (69%) had an overall PR.

Definitive therapy

Decisions regarding definitive therapy were determined on the basis of the response to induction by a multidisciplinary team of physicians. Nineteen patients (49%) received concurrent chemotherapy and RT (16 hyperfractionated (31) and 3 once-daily fractionation); 16 (41%) patients received RT alone (11 hyperfractionated and 5 once-daily fractionation); and 4 patients (10%) underwent surgery (1 patient received additional preoperative RT). Definitive RT began 3 weeks after the last dose of chemotherapy and LDFRT in all patients receiving RT or concurrent RT and chemotherapy. In general, patients tolerated induction therapy well, and it was our observation that neoadjuvant therapy did not limit the subsequent concurrent chemotherapy and RT, although the number of patients who received the combined modality approach was too small for statistical comparison ($n = 19$).

Radiotherapy was given without treatment interruptions,

Table 4. Response to LDFRT, carboplatin, and paclitaxel at primary and nodal sites

Response	*n*	CR (%)	PR (%)	SD (%)	PD (%)	RR (%)
Primary site	39	11 (29)	24 (62)	4 (10)	0	35 (90)
Neck	32	10 (31)	12 (38)	9 (28)	1 (3)	22 (69)
Overall	39	5 (13)	27 (69)	6 (15)	1 (3)	32 (82)

Abbreviations: CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease; RR = response rate.

Table 5. Comparison of response to induction therapy with carboplatin and paclitaxel

	Stage (%)				Dose		Primary site response (%)					Neck response (%)				
First author	2	3	4	Cy	C (AUC)	P (mg/m^2)	CR	PR	SD	PD	RR	CR	PR	SD	PD	RR
Dunphy (22) (n = 62)	4	19	76	3	7.5	150–265	34	32	18	16	66	33	21	NR	NR	53
Bouillet (24) (n = 20)	NR	NR	NR	2	6	175	0	55	40	5	55	NR	NR	NR	NR	NR
Machtay (23) (n = 53)	0	35	65	2	6	200	13	76	NR	NR	89	NR	NR	NR	NR	NR
Vokes† (15) (n = 69)	0	4	96	2	2* (6)	135* (405)	30/35	45/57	3/3	3/3	75/87	NR	NR	NR	NR	NR
Present study (n = 39)	0	23	77	2	6	225	28	62	10	0	90	31	38	28	3	69

Abbreviations: Cy = cycles; C = carboplatin; P = paclitaxel; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease; RR = response rate; NR = not reported.

* Weekly doses; total for 3 weeks in parentheses.

† All patients/assessable patients only.

except in 1 patient who refused RT after 4000 cGy. The total dose of RT used for definitive therapy ranged from 6600 to 7000 cGy for once-daily RT at 180–200 cGy/fraction and from 7440 to 7680 cGy for twice-daily RT at 120 cGy/fraction. In calculating the planned total dose of RT to be used for definitive therapy, the radiation oncologist incorporated the induction dose used into the final calculation for a maximal total dose (induction plus definitive) of approximately 7640 cGy (once-daily fractionation) or 8320 cGy (twice-daily fractionation).

Patient status

Thirty-one patients (79.5%) were alive and well and 1 patient (2.5%) was alive with disease at last follow-up; 1 patient (2.5%) had died of other causes, and 6 patients (15.5%) subsequently died of PD at 4, 6, 8, 11, 11, and 12 months after definitive therapy. Of those with recurrence, 5 patients (2, oropharynx; 2, oral cavity; and 1, hypopharynx) had only a PR to induction therapy, and 2 (1, oral cavity and 1, oropharynx) had SD during induction therapy. The patterns of recurrence included seven locoregional failures and three distant metastatic failures. No patients with a CR to induction had developed a relapse at the last follow-up examination.

DISCUSSION

This is the first clinical report of LDFRT (ultrafractionation) and chemotherapy given as induction therapy for SCCHN in human subjects. Chemopotentiation in this setting is novel and shifts the paradigm of RT by successfully applying a dose range previously thought to be ineffective. The preliminary results have indicated that using RT to enhance the effect of chemotherapy is tolerable and provides an excellent response rate (90% at the primary site, 69% in the neck and 82% overall). Although the median follow-up was 18 months, the emerging patterns of failure indicate greater locoregional than distant metastatic failure using this induction scheme, as seen by others (9, 10, 15).

This combination of LDFRT, carboplatin, and paclitaxel was extremely well tolerated, with toxicity comparable to that after carboplatin and paclitaxel alone in a similar patient population (22, 23). No unexpected adverse events occurred and no evidence was found that LDFRT increased the rate of radiation-induced Grade 3-4 toxicity (i.e., no excessive mucositis, esophagitis, or skin reactions in the RT port) during induction or subsequent therapy. In addition, definitive therapy did not have to be delayed (in the case of RT or surgery) or interrupted (in the case of RT), and it did not affect the tolerability of subsequent therapy. With a median follow-up of 18 months, it would be premature to report any late radiation toxicity data; however, the data collection is ongoing. Improvement in the overall survival and response to definitive therapy is the subject of long-term follow-up of these patients.

Table 5 outlines a comparison of response at the primary site and neck in published trials using paclitaxel and carboplatin as induction therapy in head-and-neck cancer (15, 22–24). When comparing these to the present study, several important points emerge. Dunphy *et al.* (22) used three cycles of carboplatin and paclitaxel in a dose-escalation study and reported a response rate of 66%. Although Dunphy *et al.* included Stage II patients in their trial and used three cycles of chemotherapy, the present trial had a better response rate in more advanced-stage patients using only two cycles of therapy. Additionally, Dunphy *et al.* (22) reported a 16% PD rate at the primary site, and we saw no progression at the primary site, using stringent evaluation criteria, and only 3% progression in the neck. The use of LDFRT with paclitaxel and carboplatin also appeared to enhance the CR rate at the primary site compared with the findings of the study by Machtay *et al.*, who used two cycles of chemotherapy alone at similar doses.

In the recently reported induction regimen by Vokes *et al.*

(15), the authors reported a response rate of 75% or 87% (all patients or assessable patients, respectively). Interestingly, 14 (20%) of 69 patients had undergone neck dissection or primary surgery plus neck dissection before induction, making interpretation of their impressive RR difficult. Carboplatin and paclitaxel were given weekly to increase dose intensity, with 80% of patients receiving >75% of the intended dose. Despite the dose density of the study by Vokes *et al.* (15), our results appear equivalent, and the ease of delivery of LDFRT, carboplatin, and paclitaxel has appeal. When comparing the present clinical trial with other neoadjuvant strategies that used three- and four-drug chemotherapy regimens (15, 16, 18, 32, 33), chemotherapy and LDFRT provided similar clinical responses at the primary site, with much less toxicity. Induction trials do not consistently report the nodal response (15, 23, 24) or fail to report SD or PD (23) rates. We believe the nodal RR of 69% is remarkable and may be attributed to the use of LDFRT in this population of patients. The notable RR in the present study suggests that LDFRT enhanced the effectiveness of chemotherapy without necessitating dose intensification.

The use of LDFRT with chemotherapy provides a new way to maximize tumor downstaging through the use of RT as a biologic modifier of the chemotherapy response. In some regard, LDFRT should be viewed as the third "antineoplastic agent" in this chemotherapy regimen. Because it is being used in a low-dose fashion, RT has a different purpose and application than its traditional role as definitive therapy. It provides identification of sensitive tumors that might respond to lower doses of definitive RT or less morbid surgery in subsequent treatment of these patients. It also confers the opportunity for improved organ preservation and/or targeted RT in this population of patients. For example, this approach may provide a way to decrease the boost phase of definitive RT given for advanced SCCHN in the future, similar to the paradigm shift seen in small-cell lung cancer (34).

The mechanism for this cellular response to low-dose RT is incompletely understood. Beyond very low doses of RT (>50 cGy), a relative increase occurs in the resistance to cell killing by RT, termed induced radiation resistance (35). The development of induced radiation resistance is dependent on intact DNA repair mechanisms (26), and the induction of DNA repair pathways after DNA damage by RT may be the regulator of induced radiation resistance. The HRS response is independent of the DNA-dependent protein kinase complex used to repair double-stranded DNA damage (26). This suggests that the HRS phenomenon is not dependent on DNA repair mechanisms (26) and that the use of LDFRT may selectively favor pro-apoptotic pathways (27). Therefore, HRS may provide a way to exploit radiation cell killing, without inducing DNA repair, thus providing a way to avoid the development of radiation resistance. Additional exploration of the mechanism through which LDFRT exerts its effect is ongoing at our institution (36, 37) and others.

As investigations in locally advanced SCCHN progress, building on the success of concurrent chemotherapy and RT, it is important to develop induction schemes that are tolerable and have a high response rate to combat the emergence of distant metastasis. Although the long-term effect on the patterns of failure is unknown, only three of the seven recurrences in our study included distant metastasis and only 1 patient had distant metastasis without locoregional failure. Equally important is the ability to maximize response and prevent the delay or decrease in dose of definitive therapy. The current induction scheme did not delay definitive RT or surgery and had a response rate of 90% at the primary site and an overall response rate of 82%. Because the precise use of LDFRT in the induction setting is still being defined, a dose "de-escalation" project is planned in head-and-neck cancer combining a novel weekly chemotherapy schedule (38) with lower doses of LDFRT to investigate the most efficacious dose and schedule of LDFRT in this patient population. If this approach provides improved CR rates in the primary site, the optimal next step would be a Phase III trial to confirm these results in a randomized, controlled fashion comparing induction chemotherapy in the presence and absence of LDFRT.

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Durable Local Responses With Subtherapeutic Doses of Concurrent Radiation and Gemcitabine in a Patient With Refractory Hodgkin's Disease

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Patient and Method

37-year-old male with recurrent nodular sclerosing Hodgkin's disease (HD), initially diagnosed in June 1989 and treated with adriamycin/bleomycin/vinblastine/dacarbazine (ABVD) chemotherapy in Greece. He was evaluated at the University of Kentucky (UK) in July 1990 for recurrent disease. He had presented with right cervical lymphadenopathy, and a biopsy was positive for HD. His bone marrow exam was negative. Computed tomographic (CT) scans showed nodal disease in the chest and abdomen. He received 5 cycles of mechlorethamine/vincristine/procarbazine/prednisone (MOPP) alternating with ABVD in 1991–92. After his chemotherapy he had persistent chest adenopathy on CT scans but he was presumed free of disease, as his bone and gallium scans were negative and he was totally asymptomatic.

He was first seen by the bone marrow transplant (BMT) service at UK in late 1995 for a second relapse. He had presented with right hip pain and adenopathy in right groin and left neck, and biopsy of a right inguinal lymph node confirmed recurrent HD. CT scans showed worsening mediastinal adenopathy. Therefore, he underwent bone marrow transplantation in early 1997 after conditioning with busulfan and cyclophosphamide; the transplantation was delayed due to problems with his insurance coverage. His posttransplant course was uneventful with apparent complete clinical response.

He was seen again by the BMT service at UK in November 1997 for a third relapse with worsening generalized adenopathy. CT scans confirmed new axillary adenopathy, 5 hypodense areas in the liver and increasing multiple splenic lesions, increased inguinal lymphadenopathy bilaterally and a lytic lesion in the T-4 vertebral body. Biopsy of a left inguinal node showed nodular sclerosing HD. In December 1997 he was placed on single-agent vinblastine chemotherapy without benefit. Later the same month he was changed to oral etoposide. This resulted in slow improvement in cervical lymphadenopathy and to a lesser degree in the axillary and inguinal nodes and total disappearance of liver lesions, but persistent lymphadenopathy was noted on CT scans. In February 1999 the etoposide was discontinued due to complaints of upper and lower extremity numbness along with heartburn.

He received navelbine every 2 weeks for 5 months starting in September 1999 for increasing anemia, night sweats, and increasing adenopathy. This initially resulted in improvement of his symptoms. However, repeat CT scans in February 2000 for increasing peripheral adenopathy confirmed increased bilateral inguinal adenopathy as well as splenomegaly. From March through April 2000, he received radiation therapy to the left axilla and pelvis at 6MV x-rays, 3960 cGy/22 fractions for painful adenopathy.

In September 2000, he presented with back pain and increasing left supraclavicular lymph nodes. A restaging workup with CT scans of abdomen and pelvis, magnetic resonance imaging (MRI) of the thoracic and cervical spine, and a bone marrow biopsy with aspirate was performed. CT scan of abdomen and pelvis with contrast showed persistent mediastinal and inguinal adenopathy, splenomegaly, increased gastro-hepatic ligament lymphadenopathy, lytic lesions of the right ileum, and multiple areas of sclerosis throughout the vertebral bodies consistent with bony involvement by HD. This was confirmed on MRIs, which showed diffuse involvement of all the cervical and thoracic vertebrae. Bone marrow biopsy showed a virtually acellular marrow (<5% cellularity) but no evidence of involvement by HD.

Due to the patient's virtually acellular bone marrow, it was felt that he was not a candidate for full-dose radiation to his spine or full-dose chemotherapy. Therefore, he was treated with low-dose radiation therapy at 60 cGy twice a day for 2 days each week to the whole spine and both supraclavicular fossae, along with gemcitabine at 625 mg/m^2 weekly, for 3 weeks. He responded well to this treatment with complete resolution of his symptoms of shoulders and back pain, although the last dose was delayed by a week due to complaints of low-grade fever. He had stable counts and negative blood, urine, throat, and viral cultures, and his symptoms were successfully treated with a course of oral antibiotics.

In December 2000 he was re-treated with the same regimen with radiation therapy to the right axilla and posterior neck for worsening painful lymphadenopathy in these regions. This time he received only 2 doses of gemcitabine, with a reduced second dose at 337.5 mg/m^2 due to thrombocytopenia. His total radiotherapy dose was also reduced to a total of 420 cGy and he was transfused 2 units of packed red blood cells for anemia. He again had complete resolution of symptoms.

In March 2001 he relapsed with pain in the left pelvis and inguinal region. MRI showed progression of disease regionally. He was treated with a total radiation dose of 640 cGy to the left pelvis given twice a day in fractions of 60 cGy for 2 days each week, along with 2 weekly doses of gemcitabine. The first dose was given at 675mg/m^2, but the second dose was reduced by 50% due to thrombocytopenia. He was again transfused 2 units of packed red blood cells for anemia.

In May 2001 he underwent splenectomy for persistent splenomegaly. Surgical pathology confirmed diffuse involvement of spleen by HD.

In June 2001 he presented with right scapular area pain. On physical exam he had an area of tenderness over right scapula. He was treated with 480 cGy of radiation to the right scapular area given in the same fashion as above. He also received 2 weekly doses of gemcitabine at 675 mg/m^2.

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He seemed to tolerate this well without any side effects and again had complete resolution of his symptoms.

In August 2001 he presented with bilateral low back pain in the areas of sacro-iliac (SI) joints. MRI showed lytic defects adjacent to SI joints consistent with bone involvement by HD. He received a total dose of 720 cGy of radiation to areas around both SI joints in the same fashion as before, along with 3 weekly doses of gemcitabine at 500 mg/m^2, without any complications.

He presented with pain and swelling of left lower extremity in January 2002. Radiological exam of the left femur showed an area of sclerosis with cortical thickening in the mid-shaft of the femur consistent with metastatic lesion from HD. He was again treated with 720 cGy of radiation given as before, along with 3 weekly doses of gemcitabine at 500 mg/m^2, without any complications again.

Since then he has not required any more treatments and has remained totally asymptomatic. He has been treated with this regimen on an as-needed basis for the last 27 months with excellent palliation of his symptoms; he has not required any treatments for the last 10 months and continues to enjoy an excellent quality of life and performance status.

Results

The patient has experienced durable comprehensive local remissions each time he has received concurrent radiation with gemcitabine; all his relapses have been at sites not previously radiated. He has only been treated when he has presented with symptoms of pain not controlled with pain medications, secondary to skeletal or soft tissue involvement. He has tolerated this therapy very well and, despite having virtually acellular bone marrow, he has not required any blood product or cytokine support, nor has his therapy been delayed or reduced due to toxicity since undergoing splenectomy more then a year ago. Prior to splenectomy he did have episodes of anemia and thrombocytopenia. He received a total of 4 units of packed red blood cells from September 2000 to March 2001. His therapy was also delayed or reduced on 3 occasions due to bone marrow toxicity. Despite dose reductions and/or delays in therapy, he still had complete resolution of symptoms with excellent local response each time he was treated. His symptoms have been effectively controlled for more then 2 years now with this combination, used only when needed. He continues to enjoy an excellent quality of life with a Karnofsky performance score of 100%, despite not receiving any treatments for the last 10 months.

Discussion

The majority of patients with early[1-3] or advanced[4-6] HD are cured or achieve long-term disease-free survival with chemotherapy alone or by combination of chemotherapy with radiation therapy.[7-10] Commonly used combination chemotherapy regimens such as MOPP/ABVD can result in remission rates of up to 80% in the treatment of HD.[11-14] Even in advanced disease, initial complete remission rates of 90% have been reported with aggressive chemotherapy regimens like BEACOPP.[15] But the treatment of relapsed or refractory HD remains a challenge despite the development of many polychemotherapy regimens for salvage therapy.[16-19] Currently, peripheral stem cell support or autologous bone marrow transplant after high-dose chemotherapy is considered the standard of care for relapsed or refractory HD.[20-24] Currently available therapies are not very effective for the treatment of patients in first relapse, with less then 25% of patients in first relapse being cured of their disease.[25,26] Furthermore these regimens generally are not easily tolerable due to severe side effects such as infertility, cardiomyopathy, and second malignancies.

Therefore a search for alternative strategies to improve the outcome of patients with relapsed HD has resulted in the use of gemcitabine.[27] This agent has been shown to be effective in heavily pretreated patients with HD who have refractory or relapsed disease.[28-30] A review of the literature showed that gemcitabine has been used at doses ranging from 1,000 mg/m^2 to 1,500 mg/m^2 given weekly, in chemotherapy cycles ranging from 3 to 7 weeks, with 1-week breaks between cycles.[28-30] We also found that although gemcitabine has acceptable toxicity, dose reductions and delays are required in one third of treatments.[29]

Gemcitabine is a pyrimidine antimetabolite with unique metabolic properties, and has proven to be very active against solid tumors as well as leukemia and lymphoma cell lines.[31-33] Gemcitabine is also a well recognized radiosensitizer, with activity against a wide variety of human tumor cells in culture.[34-36] The use of chemotherapeutic agents as radiation sensitizers[37-39] or low-dose radiation (50-80 cGy) as a chemo-sensitizer[40,41] is a relatively new concept in cancer therapy. Laboratory data suggest that tumor cells exhibit irradiation hypersensitivity at very low doses.[42-44] As previously reported, we have found that when these doses are combined with chemotherapy, a cell-killing enhancement factor of 3–4 can be achieved.[45] Gemcitabine has proven to be a potent radiosensitizer in both laboratory and clinical studies.[34-36,46,47]

In our patient with refractory HD, the use of subtherapeutic doses of gemcitabine and low-dose radiation successfully elicited durable long-term responses. This therapy was chosen for this patient as he was not a candidate for conventional dose radiation or full-dose chemotherapy due to severely depleted bone marrow reserves from multiple prior treatments for recurrent extensive disease. He had relapsed with metastatic disease to the bone, confirmed by CT and MRI exams. Both MRI and CT are known to allow excellent assessment of tumoral bone invasion by HD.[48] Involvement of the skeletal system is a rare but known complication of refractory HD; it most commonly presents with localized pain around the involved bones.[49] The vertebral column is the most frequent site of axial involvement, and the femur is the most common site overall. The most frequent radiographic pattern reported is vertebral sclerosis; other common radiological findings include periosteal reaction and hypertrophic pulmonary osteoarthropathy.[49] Osteosclerotic, osteolytic, and mixed lytic/sclerotic patterns have also been described.[50] Multiple bone involvement is the most significant prognostic factor, and concurrent extraskeletal involvement is also known to be associated with significantly decreased survival.[51]

As stated above, gemcitabine has been used for refractory HD, and gemcitabine in combination with radiation is a common treatment approach for solid tumors. A literature review did not reveal any reported cases where gemcitabine was used in combination with radiation therapy for the treatment of HD, and to our knowledge, this is the first reported case of concurrent radiation and gemcitabine for refractory HD. The patient has responded remarkably well to this therapy each time he has received it, with hardly any regimen-related toxicity. He has been treated with this regimen on an as-needed basis for the last 27 months with excellent palliation of his symptoms and has not required any treatments for the last 10 months. He has remained pain free and without any signs or symptoms of a relapse for the last 10 months, with an excellent quality of life. This case is a good example of a basic research concept initially tested in our research labs and then successfully translated to a clinical application.

The most effective and well tolerated dose of radiation in our patient was 720 cGy given in fractions of 60 cGy each twice a day for 2 days on a weekly basis for 3 weeks, concurrently with gemcitabine at 500 mg/m^2 weekly for 3 weeks. However, further studies need to be done to confirm the optimal regimen. Therefore we plan to start a phase I trial at our institute soon to determine the optimal dose and schedule for the concurrent use of radiation and gemcitabine in patients with refractory or

relapsed HD who are not able to tolerate conventional doses of radiation or chemotherapy due to certain pre-existing conditions, such as limited bone marrow reserves, poor medical or performance status.

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Review

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Using modern treatments, Hodgkin's disease (HD) is curable in 75% of patients. Unfortunately, relapsing HD after an autologous stem cell transplant (ASCT) is almost always fatal, and good palliation becomes the goal in some of those patients. The case presented by Halepota et al is the only one described using concomitant low-dose radiation and gemcitabine in a patient with recurrent HD after ASCT failure. In general, post-ASCT management is often limited by poor bone marrow reserve, and includes the use of single, sequential, and multiagent chemotherapy, as well as steroids. Local radiation therapy has been effectively used to control symptomatic localized progression.

Patients with relapsing HD after ASCT may benefit from a second transplant. Data about the use of second autotransplant is limited to a few patients. Ahmed et al[1] planned double autotransplant in refractory HD patients, showing similar results to patients with chemo-sensitive disease. The European Blood and Bone Marrow Registry reported 12 HD patients who had a second transplant after relapsing from a previous ASCT.[2] After a median follow-up of 18.5 months, 6 of 12 patients died of HD, and 2 died of toxicity, indicating, in this selective group of patients, that a second transplant could be done with acceptable toxicity. Lin et al[3] reported 3 of 5 patients alive and in remission after their second transplant. Allogeneic transplant has been used in relapsed and refractory HD, with decrease in the relapse rate but high treatment-related mortality: reported survival rates range from 20% to 24.7%, and treatment-related mortality ranges from 51.7% to 61%.[4-6] It is possible that, of those patients treated with allogeneic transplantation, only a few had failed ASCT.

The use of nonmyeloablative regimens followed by allogeneic transplantation is becoming an important investigational treatment option in hematological malignancies. Reported survival rates for HD patients, including many who had failed previous autotransplant, range from 50% to 100% at short follow-up.[7-13] Treatment-related mortality ranges from 0% to 33% in these reports, an improvement compared to the "full" allogeneic transplants. More recently, nonmyeloablative regimens have been used with matched unrelated donors.

Unfortunately, many patients will not have donors and will be treated with single-agent chemotherapy such as vinorelbine, gemcitabine, etoposide, and vinblastine. Combination therapy, such as cytarabine-cisplatin–containing regimens, can be attempted in selected patients with good bone marrow reserve. New investigational agents are currently being studied, such as liposomal vincristine, arsenic trioxide, and rituximab in patients with CD20-positive Reed-Stemberg cells. Other monoclonal antibodies, such as anti-CD30, bispecific CD30, and radioimmunoisotopes, are also being studied.

Radiation therapy has been used for relapsed HD treated with chemotherapy. Long-term remissions have been reported in some patients with localized relapse in nonirradiated areas.[14] After ASCT failure, radiation therapy is primarily palliative in nature. Gemcitabine is a pyrimidine antimetabolite that has been shown to have activity in recurrent HD.[15-17] This drug, active in lung cancer and pancreatic cancer, has been used as a radiosensitizing agent especially in pancreatic cancer and other gastrointestinal malignancies. No previous use of this combination has been reported in HD.

Halepota et al report on a young man with diagnosis of multiple recurrences of HD over a period of 12 years. After an initial 6-year remission, he relapsed and was treated with chemotherapy followed by high-dose chemotherapy and an ASCT. He relapsed in lymph nodes, liver, spleen, and bones less than 1 year after his transplant.

After posttransplant relapse, this patient was treated with systemic single-agent chemotherapy. He consecutively failed vinblastine, etoposide (toxicity), and vinorelbine. When the patient developed symptoms due to progressive disease, the authors treated him with low-dose radiation therapy in combination with low-dose gemcitabine, out of concern that extensive radiation in the spine and pelvis would compromise the already poor bone marrow reserve. The patient was treated at different times with different doses of radiation therapy and gemcitabine, and at some point required a splenectomy. After multiple customized local treatments, the patient has good palliation of his symptoms and disease control.

The amount of radiation necessary to control local HD may be lower than for other solid tumors. HD is a very radiosensitive disease, so enhancing radiation control with concomitant chemotherapy could be effective. Halepota et al assumed that low doses of radiation and gemcitabine would cause less bone marrow toxicity. It is possible that radiation therapy in the scapula and femur could be given without significant risk of myelosuppression since no significant bone marrow is located in those areas. However, extensive full doses of radiation in the spine and pelvis posttransplant in a patient with poor bone marrow cellularity would be of concern. Halepota et al do not mention the patient's blood counts, but apparently the main problems seen with combination treatment—thrombocytopenia and anemia—were easy to manage. While the reported approach was not standard, it effectively improved the quality of life and perhaps prolonged the life of this patient.

If this patient is now in complete remission, he should be evaluated for a nonmyeloablative allogeneic transplant, as he will most likely relapse of his disease at some point. The graft-versus-lymphoma effect seen with this treatment modality might enable long-term control of his disease.

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