Low-dose fractionated radiation potentiate the effects of Cisplatin independent of the hyper-radiation sensitivity in human lung cancer cells

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Abbreviation list: CER-Cisplatin enhancement ratio; FDR-False discovery rate; HRS-Hyper-radiation sensitivity; IRR-Induced radiation resistance; LDFRT-Low dose fractionated radiation therapy; NSCLC-non-small cell lung cancer; SF2-Surviving fraction at 2Gy; TUNEL-terminal transferase mediated fluorescein-dUTP end labeling.

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ABSTRACT

Purpose: In this study, the role of hyper-radiation sensitivity (HRS) in potentiating the effects of cisplatin by low dose fractionated radiation (LDFRT) was evaluated in four human non-small cell lung cancer cell lines. Experimental Design: Presence of HRS and cisplatin enhancement ratio (CER) by LDFRT/2Gy was assessed using colony-forming and apoptotic assays. Cell cycle disturbances were studied by flow-cytometry. Expression of genes involved in apoptosis was assessed using real-time RT-PCR arrays. Results: H-157 cells showed a distinct HRS region followed by UKY-29 and A549 cells while it was absent in H460 cells. H460 cells that lack HRS showed maximum CER with LDFRT (4x0.5Gy) both by clonogenic inhibition and apoptosis compared to single fraction of 2Gy while the most radio-resistant A549 cells had the least CER with no significant differences between LDFRT or 2Gy. Interestingly, in H157 cells a more pronounced CER was observed with LDFRT when assessed by apoptosis but by clonogenic inhibition CER was higher with 2Gy than LDFRT. Excluding H-157 cells, the CER by LDFRT was inversely proportional to radioresistance (determined by $D_0$ or $SF_2$) of the cells. LDFRT alone or in combination with cisplatin induced larger number of pro-apoptotic genes than 2Gy or cisplatin+2Gy in cells showing HRS when compared to H460 cells. Conclusion: These findings indicate that chemo-potentiation by LDFRT is correlated more with the intrinsic radiation sensitivity of the non-small lung cancer cells than the HRS phenomenon while the mode of cell killing is both through apoptosis and clonogenic inhibition.
INTRODUCTION

Combined treatment with radiotherapy and chemotherapy has been extensively used in the management of many types of solid malignancies including non-small cell lung cancer (NSCLC) (1, 2). This approach in NSCLC has demonstrated increased response rate, survival and local control rate (1, 3, 4). Cis-diamminedichloroplatinum (5) (cisplatin) is one of the most widely used chemotherapeutic agents. While several studies have shown cisplatin as a radio-sensitizer (6-9), other studies have reported lack of radio-sensitization activity (10, 11), or increased patient survival with high toxicity (8).

Molecular mechanisms of radio-sensitization by cisplatin are not completely understood. However, inhibition of repair of radiation-induced DNA damage by cisplatin is thought to be the underlying mechanism (12, 13). Cisplatin has been shown to prevent the translocation of Ku protein along DNA which affects non-homologous end joining pathway thus contributing to cell death (14). There are reports demonstrating that cisplatin treatment can increase DNA repair and enhance survival of cells after radiation exposure (15, 16).

The dual clinical objectives of the combined modality treatment, improved local control and prevention of distant micrometastases are constrained by the intrinsic radiation- and chemo- resistance of solid cancers causing profound patient morbidity. Recently, both in vitro and in vivo studies have demonstrated that low doses of fractionated radiation can be used as enhancers (potentiator) of full dose chemotherapy and circumvent the development of resistance observed with standard clinical doses of radiation and chemotherapy (17-20). The effects of low dose fractionated irradiation on cell survival have been now well studied and the phenomena of hyper-radiation
sensitivity (HRS) at doses of < 0.5Gy and induced radiation resistance (IRR) at doses of >1Gy are well documented (15, 21). HRS does not induce cellular repair mechanisms often observed at clinically relevant or higher radiation doses and thus provides a plausible explanation as to why there is no induction of radiation resistance with HRS as measured in vitro (21). Further, HRS inducing low dose failed to induce pro-survival transcription factors such as NFκB and NF-Y that is necessary for increasing the levels of MDR-1 gene (22). Hence, low or no MDR-1 induction in response to LDFRT will help to enhance the effects of chemotherapy that is often mitigated by MDR-1. Critically, to take advantage of the benefits of HRS in the clinical setting, radiotherapy would have to be extended over 7-12 weeks (ultrafractionation) to reach a total dose equivalent to that of standard radiation therapy approaches. Realistically, prolongation of radiation therapy using LDFRT in clinical settings is not possible due to the cost factor as well as biologically there is no clear evidence whether such HRS phenomenon exists in vivo. In order to avoid such issues, the enhanced cell killing effects of low dose fractionated radiation therapy (LDFRT) in the form of HRS can be exploited by combining it with chemotherapy. The present study investigates the effects of cisplatin in NSCLC cell lines, H-157, H460, A549 and UKY-29, in combination with LDFRT as well as conventional dose of radiation. Studies were designed to understand the mechanisms underlying cisplatin-potentiation effects by LDFRT as well as the role of HRS in determining the cellular response and mode of cell killing. The findings indicate that chemo-potentiation by LDFRT is correlated more with the intrinsic radiation sensitivity of the cells than the HRS phenomenon while the cell killing is rendered either by apoptosis or clonogenic inhibition.
MATERIAL AND METHODS

Cell Culture

Four established NSCLC cell lines, H-157, H460, A549 and UKY-29 were used for the present studies. H-157, H-460 cells were provided by Dr. John Yannelli, University of Kentucky in 2003 and UKY-29 cell line was established using pleural effusion sample from a lung cancer patient at University of Kentucky, Lexington, KY (23). A549 cells were obtained from American Type Culture Collection (ATCC) in 2003. Cell lines were tested and authenticated by RADIL (Columbia, MO) using short tandem repeat (STR) markers. The alleles for 9 different STR markers were determined for each sample and the results were compared to the genetic profiles reported by ATCC for each cell line in May, 2010. No cross-contamination with other species was observed (Supplementary Table 1). All cell lines were cultured at 37°C and 5% CO₂ in RPMI supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cell Treatments

Cells were treated with cisplatin (Bedford Laboratories, Bedford, OH) formulated in 0.9% sodium chloride at a stock concentration of 1mg/ml. A 100kV industrial X-ray machine (Phillips, Hamburg, Germany) was used to irradiate the cells at room temperature. The dose rate with a 2mm Al plus 1mm Be filter was ~2.64Gy/min at a focus surface distance of 10.5cm.

Treatments were performed in the following manner; (1) left untreated (UT); (2) 0.1Gy to 6Gy dose of radiation; (3) cisplatin (for H-157, 0.025μg/ml to 0.5μg/ml; for UKY-29, 0.01μg/ml to 0.5μg/ml; for both H460 and A549, 0.01μg/ml to 0.5μg/ml). For
combined treatments (cisplatin plus radiation), the cells were treated with cisplatin (H-157, 0.04μg/ml; H460 and UKY-29, 0.25μg/ml; A549, 0.3μg/ml) and immediately exposed to radiation unless specified otherwise without changing the medium. For multi-fractionated experiments, cells were exposed to radiation without changing the medium at 0.5Gy in four fractions, with 8h time intervals between each fraction. In a separate set of experiments, cells were exposed to a total dose of 6Gy (2Gy, 1Gy and 0.5Gy fractions) using an 8h time interval between each fraction.

**Colony forming assay**

Clonogenic survival assays were performed for each treatment group as described previously (17, 18, 24). Briefly, for each treatment, cells were plated in two different cell concentrations in quadruplet sets (total 4 replicates). After overnight plating, cells were treated as explained above and left for colony formation. After incubation for 10 or more days, colonies were stained with crystal violet and the colonies containing more than 50 cells were counted. The surviving fraction (S.F.) was calculated as a ratio between the number of colonies formed and the product of the number of cells plated and the plating efficiency. A plating efficiency (PE) of 60-80% was obtained depending on the cell line. \( D_0 \) values were determined using single-hit multi-target model. Cisplatin enhancement ratio (CER) by radiation was calculated using the following formula based on surviving fraction (SF):

\[
CER = \frac{SF_{\text{cisplatin alone}}}{SF_{\text{cisplatin+ radiation}}}
\]

The radiation ER (RER) by cisplatin was calculated as follows:

\[
RER = \frac{SF_{\text{radiation alone}}}{SF_{\text{cisplatin+ radiation}}}
\]
Quantification of apoptosis

Flow-cytometric analysis:

Untreated (UT) or treated cells as described above were incubated in growth media for specific periods of time (from 0 to 48h). Adherent and floating cells were harvested by trypsinization. Flow-cytometric measurements of cellular DNA content were performed in ethanol-fixed cells using propidium iodide (PI) as described earlier (25). PI stained cellular DNA was excited at 488nm using flow-cytometer (FACS Calibur; Beckton and Dickenson, USA) and the data was acquired using the Cell Quest software (Beckton and Dickenson, USA). Apoptotic cells were quantified gating sub G0/G1 population.

TUNEL assay:

The in situ apoptosis detection kit (Roche Diagnostics Corporation, Indianapolis, IN) which detects the DNA strand breaks in single cells by terminal transferase mediated fluorescein-dUTP end labeling (TUNEL) was used as per the instructions provided by the manufacturer. Cells were seeded in chamber slides and exposed to cisplatin alone (IC50 concentrations); 2Gy, single fraction (2Gy); combination of cisplatin and 2Gy, single dose (cisplatin+2Gy); and cisplatin plus four fractions of 0.5Gy (cisplatin+LDFRT). After 24h and 48h of treatment, cells were stained using TUNEL kit. The stained slides were observed in triple band-pass filter using Nikon-microphoto epifluorescence microscope. To determine the percentage of cells showing apoptosis a total of 2000 cells were counted for each treatment.
Enhancement of cisplatin-induced apoptosis by radiation was calculated using the following formula based on percentage of induction of apoptosis (PIA):

$$CER = \frac{PIA_{\text{cisplatin+radiation}}}{PIA_{\text{cisplatin alone}}}$$

**Real time reverse transcriptase-polymerase chain reaction**

Total RNA was extracted from the cells 3h after treatment using TRIzol reagent (Life Technologies, Inc.). 1μg of total RNA was reverse transcribed into cDNA using SuperArray’s RT² first strand kit (Cat. No. C-03) and instructions provided by them. Real time RT-PCR was performed with RT² profiler PCR array for human apoptosis (SuperArray; Cat. No. APHS-012A) to test the expression levels of 84 genes (Supplementary Table 2) involved in apoptosis using Applied Biosystems 7300 instrument.

**Western blot analysis**

Total protein extracts obtained at 6h after treatment of A549 cells were subjected to Western blot analysis as previously described (26). Briefly membranes were incubated overnight at 4°C with primary antibodies against XIAP (BD Bioscience). The same membrane was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling) as previously described (27). Densitometric analysis was done using the NIH ImageJ software (http://rsb.info.nih.gov/ij/).
**Statistical methods**

Independent two-sample Student’s t-test was performed to test the differences in the means of radiation inactivation estimates and percentage of apoptosis (TUNEL-positive cells) obtained from any given two different treatment groups of cell lines. Due to repeatedly measured surviving fractions at each dose level as well as over different doses repeated measures analysis of variance to surviving fractions were used with autoregressive variance-covariance matrix. One-way analysis of variance to surviving fractions with different fractionating groups were used to determine if there were differences in mean surviving fractions among different fractionations for a given cell line. Independent two-sample Student’s t-test was performed to test whether there are any differences in the mean normalized gene expression level of any given gene between any given two cell lines at a particular treatment regimen. False discovery rate (FDR)-adjusted p-values were calculated to correct for multiple testing. Data management and the statistical analyses were performed with SAS version 9.2 for Windows (SAS Institute Inc., Cary, NC). Type-I error rate was set to 5% if not stated otherwise due to multiple testing.

**RESULTS**

*HRS phenomenon was present in H-157, UKY-29 and A549 cells but not in H460 cells*

To investigate the presence of HRS and radiation sensitivity in these cell lines, cells were irradiated at low and high doses of radiation respectively and surviving fractions were obtained by colony forming assay. The presence of HRS in H-157, UKY-29 and A549 cells at low dose radiation (0.1Gy to 1Gy) was observed as most, moderate
and least prominent phenomenon respectively (Figure 1) while H460 cells showed no HRS phenomenon (Figure 1D). These results were confirmed by statistical analysis as explained in the methods section with a hypothesis that there is no difference in slope of SF of cells between “high” and “low” doses. Overall, there were statistically significant differences in slope of SF between “high” and “low” doses for cell lines H-157, UKY-29, A459 but not for cell line H460 (Supplementary table 3).

To improve the understanding of HRS in a setting of low dose fractions versus clinically relevant dose fractionation settings, the cells were subjected to 12 fractions of 0.5Gy or 6 fractions of 1Gy or 3 fractions of 2Gy. The decrease in SF was directly proportional to the number of fractions in all the cell lines for all fractionating schemes and the differences were statistically significant (Figure 2 and supplementary table 4).

Overall, all the fractionation schemes were statistically significant and 2Gy dose per fraction had the highest SF in both H-157 and UKY-29 except at a total dose of 4Gy and 6Gy for UKY-29 (Figure 2A and B and supplementary table 4). For UKY-29, the mean estimates (of SF) at a total dose of 4Gy for 0.5Gy, 1Gy and 2Gy dose per fraction were very close to each other and resulted in no statistically significant difference at all possible pairwise comparisons (p-values ranges from 0.0331 to 0.5561). However, at a total dose of 6Gy, the mean differences between all possible pairwise comparisons were statistically significant (p-value<0.0003).

Interestingly, in A549 cells that have least HRS phenomenon and H460 cells lacking HRS, significant differences in clonogenic inhibition were not observed either with 0.5Gy or 1Gy or 2Gy fractions (data not shown).
**H-157 cells were most sensitive to cisplatin and ionizing radiation-induced clonogenic inhibition**

Predictably, continuous incubation of the cells with cisplatin resulted in decreased surviving fraction with increasing concentrations (Supplementary figure 1). The IC$_{50}$ concentrations were 0.04μg/ml for H-157, 0.25μg/ml for both UKY-29 and H460, and 0.3μg/ml for A549 cells suggesting that H-157 cells were most sensitive to cisplatin treatment and A549 were most resistant. This is similar to the data that was demonstrated above in which H-157 cells showed HRS phenomenon and overall greater sensitivity to ionizing radiation along the entire range of radiation doses (0.1Gy-6Gy) (SF$_2$=0.18; D$_0$=106cGy) when compared to UKY-29 (SF$_2$=0.37; D$_0$=149.5cGy) and A549 cells (SF$_2$=0.66; D$_0$=159.4cGy) (Figures 1A-D). This six-fold difference in sensitivity to cisplatin together with the presence of the HRS phenomenon may result in an increased chemo-potentiation. However, H460 cells that lacked HRS but with a radiation response similar to H-157 cells (SF$_2$=0.26; D$_0$=110cGy) showed cisplatin sensitivity similar to UKY-29 cells (Supplementary figure 1 and figure 1A, D) and demonstrated maximum CER by LDFRT (Figure 3).

**LDFRT chemo-potentiated the effects of cisplatin in all the cell lines**

Cisplatin-potentiating effects of either single fraction of 2Gy or LDFRT were analyzed by colony forming assays (Figure 3A). The results demonstrated that both 2Gy and LDFRT chemo-potentiate the effects of cisplatin in all the cell lines (Figure 3A). The chemo-potentiating effects of LDFRT were maximum in H460 cells with a CER of 52.4 compared to 18.5 for 2Gy single fraction followed by UKY-29 cells (Figure 3B).
differences in CER between 2Gy and LDFRT were the smallest in A549 cells (1.86 vs. 1.92). Interestingly, only H-157 cells showed enhanced chemo-potentiation with single fraction of 2Gy than LDFRT (7.1 vs. 2.9). To ascertain the role of apoptosis in chemo-potentiating effects of LDFRT, induction of apoptosis was determined by sub G_0/G_1 population assessed by flow-cytometric method (Figure 4). Similar to colony forming results, both LDFRT and 2Gy single fraction radiation resulted in CER of more than 1.0 in all the cell lines except A549 (Figure 3B). H460 cells again demonstrated maximum CER with LDFRT compared to 2Gy radiation (8.3 vs. 3.5). While, significant differences in CER were not observed in UKY-29 cells for LDFRT and 2Gy IR (1.5 vs. 1.8), H157 cells showed more CER with LDFRT than 2Gy IR (2.9 vs. 2.0). TUNEL assay for apoptosis investigated in H-157 and UKY-29 cells showed similar results (Figure 3B and Table 1).

Interestingly, chemo-potentiating effects of LDFRT were found to be dependent on the intrinsic radiation sensitivity of the cells (Figure 3C). Except H-157 that was the most radio-sensitive cell line with proficient HRS, CER was inversely proportional to radio-resistance both when determined by clonogenic inhibition or apoptosis.

*Pre-treatment of cisplatin resulted in G_2/M arrest in UKY-29 cells without significant changes in chemo-potentiating effects of LDFRT and 2Gy radiation*

Hyper-radiosensitivity, like standard cellular radiation sensitivity, is cell cycle dependent and occurs most prominently during the G_2/M phase (28). We examined the impact of cisplatin-induced cell cycle arrest as a way to explain the underlying mechanism in which LDFRT potentiates cisplatin. A transient, robust cell cycle arrest
was observed in G2/M phase at 24h after incubation with cisplatin in UKY-29 cells while this phenomenon was not observed in any other cell lines (Figure 4A vs. 4B, C, D). Thus, the effect of G2/M arrest on chemo-potentiation of cisplatin by LDFRT or single fraction of 2Gy radiation was assessed by colony forming assay following incubation of UKY-29 cells for 24h with cisplatin and compared with H-157 cells. There were no significant changes in the CER in UKY-29 cells (7.1 vs. 6.4 for 2Gy and 10.2 vs. 10.2 for LDFRT); however, chemo-potentiation of cisplatin by LDFRT and 2Gy was diminished in H-157 cells (7.1 vs. 5.0 for 2Gy and 2.9 vs. 1.6 for LDFRT (Supplementary table 5). The decrease in CER by prior incubation of H-157 cells with cisplatin might be due to the induction of robust repair mechanisms that will interfere with lethal apoptotic response mediated by HRS.

Further, the radiation/cisplatin/combination-induced disturbances in the cell cycle distribution were investigated in all the cell lines (Figure 4). While, both H-157 and H460 cells showed a transient accumulation of cells in G2/M phase following 2Gy irradiation, H-157 cells demonstrated a G2/M block following LDFRT also. Neither single fraction of 2Gy or LDFRT induced significant changes in G2/M phase in UKY-29 and A549 cells. LDFRT however induced G0/G1 arrest in A549 cells. H-157 cells demonstrated a G2/M phase block following the combined treatment with cisplatin and LDFRT with no significant changes when cisplatin was combined with 2Gy (Figure 4B). On the other hand, H460 cells showed an accumulation in G2/M followed by a block in G0/G1 in response to cisplatin and 2Gy. Combination of cisplatin with LDFRT resulted in G0/G1 block only. These cell cycle disturbances might be underlying reasons for increased apoptosis (the percent cells undergoing apoptosis are shown for the groups where
induction of apoptosis was significant) in these cells at later time points (Figure 4B and D). UKY-29 cells showed a consistent G2/M block when cisplatin was combined with 2Gy with no significant changes in response to cisplatin+LDFRT treatments (Figure 4A). In A549 cells, the combination of cisplatin and LDFRT resulted in a transient G0/G1 block (Figure 4C). In both cell lines, however, hypodiploid peak indicating apoptosis was not observed.

_enhanced expression of pro-apoptotic genes was observed in cells demonstrating HRS following LDFRT or combination treatments_

We and others have shown earlier that a significant amount of apoptosis is induced by LDFRT (17, 18, 29). To understand the mechanisms underlying chemopotentiation of cisplatin by LDFRT, real time RT-PCR was performed for 84 genes related to apoptotic pathways in the four lung cancer cell lines. Expression of genes following each treatment was compared with untreated samples for each cell line (Supplementary table 6). Expression of genes in H-157, UKY-29 and A549 cells were also compared with gene expression in H460 cells for the respective treatments groups (Figure 5A-C). Genes were thus classified as either up-regulated, down-regulated or no-change.

Although, expression of genes changed significantly compared to untreated in all of the cells, by setting the threshold as 50-fold differences in expression, only few genes showed modulated expression (Supplementary table 6). In addition to the genes listed in supplementary table 6, several other genes were modulated (less than 50 fold difference) following various treatments in all the cell lines. Interestingly, in H-157 cells (most
prominent HRS and most radiosensitive), pro-apoptotic genes, CASP5, CASP6, NOD1, CD40, CD40LG, CIDEA, CIDEB, DAPK1 and FASLG were differentially induced following LDFRT treatments. In UKY-29 cells with moderate HRS, pro-apoptotic genes, CASP10 and RIPK2 were significantly upregulated in LDFRT and cisplatin+LDFRT groups with downregulation of FADD in cisplatin+2Gy group. In A549 cells having least HRS, pro-apoptotic genes, CASP8 and DAPK1 were upregulated in LDFRT and cisplatin+LDFRT groups compared to 2Gy and cisplatin+2Gy groups. Further, pro-apoptotic genes, BAG-4, CASP10 and NOD1 were downregulated in either 2Gy or cisplatin+2Gy or both the groups. In H460 cells demonstrating no HRS, however, only TNFSF10 was up-regulated in LDFRT and cis+LDFRT groups.

In figure 5, percent of genes with statistically significant up- or down- regulated changes in expression are shown when the cells having HRS were compared with H460 cells that did not have HRS. In H-157 cells, LDFRT resulted in up-regulation of more pro-apoptotic genes as compared to H460 cells followed by cisplatin and cisplatin+2Gy treatment groups (Figure 5A). However, maximum number of anti-apoptotic genes were down-regulated in 2Gy group. In UKY-29 cells, cisplatin, 2Gy and cisplatin+2Gy groups demonstrated similar upregulation of both pro-and anti-apoptotic genes (Figure 5B). LDFRT groups showed less number of pro- or anti-apoptotic genes upregulation. In A549 cells, significant downregulation of anti-apoptotic genes was observed in cisplatin, 2Gy and cisplatin+2Gy groups (Figure 5C). No significant differences were observed in LDFRT groups. A comprehensive list is provided for the genes that were significantly modulated in H-157, UKY-29 and A549 cells compared to H460 cells in supplementary table 7. Since, A549 cells were resistant to either LDFRT or 2Gy irradiation, to establish
a causal correlation, we studied the expression of XIAP, an inhibitor of apoptosis in these cells. The XIAP was induced in A549 cells following radiation as demonstrated by Western blot analysis suggesting a causal correlation between the increased expression of XIAP and decreased clonogenic inhibition (Figure 5D). These results suggest that for certain cells that are radio-resistant, use of specific inhibitors of XIAP may be vital for enhancing cell killing with either LDFRT or 2Gy.

**DISCUSSION**

Our previous in vitro, in vivo and clinical studies have demonstrated that LDFRT can be used as a chemo-potentiator of paclitaxel, carboplatin and gemcitabine (18-20, 30). However, the question that still remains unanswered is whether presence of HRS is essential to potentiate the effects of chemotherapeutic drugs by LDFRT. Since, there are currently no isogenic in vitro HRS models available that can be utilized to delineate the mechanisms of chemopotentiation by LDFRT, in the present study four lung cancer cell lines were used that had different degree of HRS to answer this question. It was observed that H-157, UKY-29 and A549 demonstrated HRS while H460 cells did not (Figure 1). Although, the reasons for the absence of HRS in some cells are not well understood, it has been suggested that HRS-negative cell lines have a dissociation between ATM activity and early G2-phase checkpoint function, producing an aberrant early G2 checkpoint response that evades dose-dependent ATM regulatory control (31). However, H460 cells did not show any G2-phase arrest after LDFRT (Figure 4D). Therefore, some other mechanisms may be responsible in these cells for failure of exhibiting HRS.
In this study, both radio-resistant cells such as UKY-29 and A549 as well as radio-sensitive cells such as H-157 did demonstrate the presence of HRS. These observations are contrary to the reports that radio-resistant cells express increased HRS/IRR (32). In fact, the most pronounced HRS was demonstrated in the most radiosensitive H-157 cells suggesting that HRS is not an exclusive phenomenon for radio-resistant tumor cells.

The present study findings strongly suggest that LDFRT can chemo-potentiate the effects of cisplatin in H460 cells that lack HRS phenomenon as well as in H-157, UKY-29 and A549 cells harboring varying degree of HRS. Further, a higher CER was observed with LDFRT compared to single fraction of 2Gy in all the human lung cancer cell lines studied except A549 (Table 1). More importantly, LDFRT potentiated the effects of cisplatin maximally in H460 cells although they were HRS-negative. In addition, a linear relationship was observed between CER and intrinsic radio-sensitivity of these cell lines (Figure 3C). Thus, together these observations demonstrate that chemo-potentiation of cisplatin by LDFRT may be independent of HRS but depend more on intrinsic radio-sensitivity of the cells.

A differential response to different types of fractionated radiation exposures was observed in H-157 and H460 cells. HRS-positive H-157 cells were more sensitive to fractions of 0.5Gy (a total dose of 6Gy) than fractions of 1Gy or 2Gy while HRS-negative H460 cells showed no differential effect with either of these fractions (Figure 2A). These findings potentially suggest that low-dose fractionated scheme settings may be advantageous in cells that harbor HRS phenomenon.
Short et al. (33) demonstrated the influence of cell cycle phases on the HRS phenomenon. Cells synchronized in G2/M showed a significantly more pronounced HRS than synchronized cells in G1 or S. These findings present an interesting explanation of the underlying mechanisms of chemo-potentiation by LDFRT in the studies from our laboratory using paclitaxel or docetaxel (18, 19). In the present study, incubation of cells with cisplatin for 24h resulted in a transient accumulation in G2/M phase only in UKY-29 cells (Figure 4A). However, in these cells CER was not enhanced following either LDFRT or 2Gy treatment compared to when cisplatin was added immediately prior to irradiation (Supplementary table 5). This is in contrast to the earlier reports where LDFRT was able to enhance the chemo-potentiation effects of paclitaxel that arrest cells in G2/M phase (18, 19). One explanation could be that the G2/M phase arrest induced by cisplatin is similar to the classical G2/M phase block induced by radiation due to accumulation of damaged G1 and S phase cells in G2/M phase. Cells arrested in G2/M phase by this mechanism do not show HRS as only a recently described second G2/M checkpoint (34) is thought to be involved in induction of HRS (28). Further, in H-157 cells the chemo-potentiating effects were reduced when cells were incubated with cisplatin 24h prior to LDFRT although no G2/M block was observed (Figure 4B and supplementary table 5). Similar results were obtained by Marples and Skov (35) in CHO cells as cisplatin increased resistance to subsequent low-dose radiation exposures by abolishing HRS. Since, H-157 cells showed HRS, it is likely that a DNA damaging agent like cisplatin can trigger the repair mechanisms so that HRS is lost and IRR is induced when cells are exposed to subsequent doses of radiation (32, 36). Alternatively, both cisplatin and radiation may target the same subpopulation of the cells. Pretreatment, thus
eliminates the sensitive subpopulation so that the remaining cell population are more resistant to subsequent treatments (36).

Our previous studies in head and neck tumor cell lines and mice xenografts demonstrated increased apoptosis rather than clonogenic death in LDFRT mediated chemo-potentiation (18, 19) suggesting that preferred mode of killing in chemo-potentiation by LDFRT is apoptosis. This is supported by the TUNEL assay results of the present study where it was found that chemo-enhancement ratio was significantly higher for apoptosis than clonogenic inhibition in H-157 cells. A relationship between apoptosis and the prevalence of low dose HRS has been demonstrated previously (29). Hence, HRS-negative H460 cells showed increased clonogenic inhibition than apoptosis when compared to HRS-positive H-157 cells. However, UKY-29 and A549 cells harboring HRS did not demonstrate significant induction of apoptosis following LDFRT thus supporting the previous observations that apoptosis may be indicative of HRS but is not a prerequisite for HRS (37, 38). Further, in H460 cells significant differences in expression of genes related to apoptosis were not observed with any of the treatments when compared to the untreated group (Supplementary table 6). For other lung cancer cells that harbor HRS, significant changes in pro- and anti-apoptotic gene expression was observed as expected. Several pro-apoptotic genes such as BAG3, NOD1, CASP5, CASP6, etc. were induced in H-157 cells followed by UKY-29 and A549 cells. The expression of apoptotic genes thus significantly correlated with the biological response as well as with the HRS status of the cells.

Of importance, the expression of several pro-apoptotic genes was significantly higher in untreated HRS-positive A549, UKY-29 and H-157 cells compared to HRS-
negative H460 cells (data not shown). Following various treatments, a limited number of genes showed significant enhanced expression in these three HRS-positive cells compared to HRS-negative H460 cells (Supplementary table 7). Significant of them were induction of FASLG in H-157 cells by LDFRT; CASP1 in UKY-29 cells and induction of CIDEB by LDFRT and down-regulation of NOD1 by 2Gy in A549 cells.

In summary, the present findings demonstrate that LDFRT chemo-potentiates the effects of cisplatin in human lung cancer cell lines irrespective of HRS status and mode of cell killing could be either apoptosis or clonogenic inhibition. This indicates that this regimen may benefit all cases of NSCLC. However, further studies to elucidate the molecular mechanisms of chemo-potentiation of cisplatin by LDFRT are needed to fully exploit this approach as a novel therapeutic modality in the clinic for the treatment of lung cancer.

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LEGENDS TO FIGURES

Figure 1: Effects of radiation on survival of lung cancer cells assessed by colony-forming assay. Effects of X-ray irradiation (0.1Gy-6Gy) on survival in H-157 (A), UKY-29 (B), A549 (C) and H460 (D) cells by colony forming assay. The smoothed lines are determined from fitting exponential regression curves. Insets show the surviving fractions at doses from 0.1Gy to 1Gy in the above four cell lines demonstrating the HRS and IRR phenomenon. Statistical analysis for Figure 1 is shown in supplementary table 3.

Figure 2: Effects of various fractionation schema on the survival of lung cancer cells. H-157 (A) and UKY-29 (B) cells were irradiated with fractions of either 0.5Gy or 1Gy or 2Gy to a total dose of 6Gy with a time interval of 8h between each fraction. Survival was then assessed by colony forming assay. For figure clarity purposes, standard error is not shown which was less than ± 10%. Statistical analysis for Figure 2 is shown in supplementary table 4.

Figure 3: Effects of radiation combined with cisplatin on clonogenic inhibition or apoptosis of lung cancer cells. A. Cells were incubated with cisplatin (H-157, 0.04μg/ml; H460 and UKY-29, 0.25μg/ml; A549, 0.3μg/ml) and irradiated immediately with either four fractions of 0.5Gy separated by 8h or single fraction of 2Gy. Surviving fractions were determined by colony forming assays. B. Chemo-enhancement ratios calculated from the data obtained in A for 2Gy or LDFRT are shown as clonogenic inhibition-CER. Further, cells were either incubated with cisplatin alone or irradiated (LDFRT or 2Gy) or exposed to
combined treatment. After 48h, cells were fixed in 70% ethanol and stained with PI. DNA content was measured by flow-cytometry and data was analyzed for sub G0/G1 population to estimate apoptosis and CER (Flow cytometry-CER). Also, CER determined by TUNEL assay is shown as TUNEL-CER. C. CER obtained either from colony forming assay (clonogenic inhibition) or flow-cytometric analysis (apoptosis) with LDFRT is plotted against D0 or SF2 (determined from Figure 1 data using single hit multiple target model) to demonstrate the correlation between radiation sensitivity and chemo-potentiating effects of LDFRT.

**Figure 4: Cell cycle perturbations induced by cisplatin and radiation in lung cancer cells.** Flow cytometric measurement of cell cycle distribution of the cells after incubation with cisplatin (H-157, 0.04μg/ml; H460 and UKY-29, 0.25μg/ml; A549, 0.3μg/ml) followed by immediate irradiation (2Gy or LDFRT). For LDFRT, 0h represents the time after the cells were exposed to the last fraction of 0.5Gy. A. UKY-29, B. H-157, C. A549 and D. H460.

**Figure 5: Changes in expression of apoptotic genes induced by cisplatin and radiation.** Cells were treated with either cisplatin or LDFRT or 2Gy or their combination and total RNA was extracted from the cells 3h after treatment. RNA was reverse transcribed into cDNA. Real time RT-PCR was performed with RT² profiler PCR array for human apoptosis to test the expression levels of 84 genes involved in apoptosis. Gene expression was normalized with the five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, ACTB) for each treatment group. Further, ratios of treated normalized gene expression levels to
untreated normalized gene expression levels of 84 genes were used as an input data for statistical analysis. Then the expression of genes for each treatment group in H-157 (A), UKY-29 (B) and A549 (C) cells was compared with gene expression in H460 cells. Genes were functionally classified as pro- or anti-apoptotic. Percent upregulated or downregulated pro- and anti-apoptotic genes was determined and represented in the figure for the genes that differed statistically significantly in their expression. (D) XIAP immunoblot analysis of A549 protein lysates. GAPDH was used as a loading control and numbers represent densitometric analysis of XIAP normalized to GAPDH.
Table 1: Summary of radiation inactivation estimates, radiation enhancement ratios and chemotherapy enhancement ratios following colony-forming and TUNEL and flow-cytometric assays for clonogenic inhibition and apoptosis respectively in H-157, H460, UKY-29 and A549 cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HRS</th>
<th>Radio-sensitivity</th>
<th>Cisplatin sensitivity</th>
<th>Colony forming assay-CER</th>
<th>TUNEL-CER</th>
<th>Flow-cytomtery-CER</th>
<th>RER</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-157</td>
<td>Most</td>
<td>1 (0.18/106 cGy)</td>
<td>1 (0.04 µg/mL)</td>
<td>2.9</td>
<td>7.1</td>
<td>11.1</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.88</td>
<td>2</td>
<td>2.57</td>
<td></td>
</tr>
<tr>
<td>H460</td>
<td>None</td>
<td>2 (0.26/110 cGy)</td>
<td>2 (0.25 µg/mL)</td>
<td>52.4</td>
<td>18.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.3</td>
<td>3.5</td>
<td>38.9</td>
<td>11.6</td>
</tr>
<tr>
<td>UKY-29</td>
<td>Moderate</td>
<td>3 (0.37/149 cGy)</td>
<td>3 (0.25 µg/mL)</td>
<td>10.2</td>
<td>7.1</td>
<td>6.2</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>1.8</td>
<td>6.1</td>
<td>5.12</td>
</tr>
<tr>
<td>A549</td>
<td>Less</td>
<td>4 (0.66/159 cGy)</td>
<td>3 (0.3 µg/mL)</td>
<td>1.92</td>
<td>1.86</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
<td>0.8</td>
<td>2.4</td>
<td>1.93</td>
</tr>
</tbody>
</table>
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Figure 1

- **A** H-157
  - Observed: □
  - Smoothed: ○

- **B** UKY-29
  - Observed: □
  - Smoothed: ○

- **C** A549
  - Observed: □
  - Smoothed: ○

- **D** H460
  - Observed: □
  - Smoothed: ○
Figure 3
Figure 4

C

A549

UT

G$_2$/G$_1$=63%
S=16%
G$_2$/M=16%

Cis

G$_2$/G$_1$=63%
S=16%
G$_2$/M=16%

2Gy

G$_2$/G$_1$=63%
S=16%
G$_2$/M=16%

Cis+2Gy

G$_2$/G$_1$=63%
S=16%
G$_2$/M=16%

LDFRT

G$_2$/G$_1$=66%
S=11%
G$_2$/M=23%

Cis +LDFRT

G$_2$/G$_1$=58%
S=14%
G$_2$/M=27%

0 h

12 h

24 h

48 h

D

H460

UT

G$_2$/G$_1$=62%
S=18%
G$_2$/M=19%

Cis

G$_2$/G$_1$=62%
S=18%
G$_2$/M=19%

2Gy

G$_2$/G$_1$=62%
S=18%
G$_2$/M=19%

Cis+2Gy

G$_2$/G$_1$=62%
S=18%
G$_2$/M=19%

LDFRT

G$_2$/G$_1$=71%
S=7%
G$_2$/M=18%

Cis +LDFRT

G$_2$/G$_1$=71%
S=7%
G$_2$/M=18%

0 h

12 h

24 h

48 h

Relative DNA content
Molecular Cancer Therapeutics

Low-dose fractionated radiation potentiate the effects of Cisplatin independent of the hyper-radiation sensitivity in human lung cancer cells

Seema Gupta, Toru Koru-Sengul, Susanne M. Arnold, et al.

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