Title

Biologically Augmenting Radiation Therapy by Inhibiting TGFβ in NSCLC from Molecular to Microenvironment

Journal

International Journal of Radiation Oncology • Biology • Physics, 87(2)

ISSN

0360-3016

Authors

Du, S
Pellicciotta, I
Barcellos-Hoff, M

Publication Date

2013-10-01

DOI

10.1016/j.ijrobp.2013.06.359

Peer reviewed

Elsevier Editorial System(tm) for International Journal of Radiation Oncology*Biology*Physics
Manuscript Draft

Manuscript Number: ROB-D-14-00878R1

Title: TGFβ Inhibitors Enhances Radiation Sensitivity of NSCLC Cells In Vitro and In Vivo by Attenuating the DNA Damage Response

Article Type: Full Length Article

Section/Category: Biology Contribution

Corresponding Author: Dr. Mary Helen Barcellos-Hoff, PhD

Corresponding Author's Institution: New York University

First Author: Shisuo Du, M.D, PhD.

Order of Authors: Shisuo Du, M.D, PhD.; Sophie Bouquet, PhD.; Chen-Hao Lo, M.S.; Ilenia Pellicciotta, PhD.; Shiva Boloruchi, PhD. Candidate; Renate Parry, PhD.; Mary Helen Barcellos-Hoff, PhD

Abstract: Purpose To determine whether TGFβ inhibition increases the response to radiotherapy in human and mouse non-small cell lung carcinoma (NSCLC) cells in vitro and in vivo.
Methods TGFβ mediated growth response and pathway activation were examined in human NSCLC NCI-H1299, NCI-H292, and A549 cell lines and murine Lewis lung cancer (LLC) cells. Cells were treated in vitro with LY364947, a small molecule inhibitor of the TGFβ type I receptor kinase, or with pan-isoform TGFβ neutralizing monoclonal antibody, 1D11, before radiation exposure. DNA damage response was measured by ATM or Trp53 protein phosphorylation, γH2AX foci formation or comet assay in irradiated cells. Radiation sensitivity was determined by clonogenic assay. Mice bearing LLC syngeneic subcutaneous tumors were treated with 5 fractions of 6 Gy and/or neutralizing or control antibody.
Results NCI-H1299, A549 and LLC NSCLC cell lines pretreated with LY364947 prior to radiation exposure exhibited compromised DNA damage response indicated by decreased ATM and p53 phosphorylation, reduced γH2AX foci, and increased radiosensitivity. NCI-H292 cells were unresponsive. TGFβ signaling inhibition in irradiated LLC cells resulted in unresolved DNA damage. Subcutaneous LLC tumors in mice treated with TGFβ neutralizing antibody exhibited fewer γH2AX foci after irradiation and significantly greater tumor growth delay in combination with fractionated radiation.
Conclusions TGFβ inhibition prior to radiation attenuated DNA damage recognition and increased radiosensitivity in most NSCLC in vitro and promoted radiation-induced tumor control in vivo. These data support the rationale for concurrent TGFβ inhibition and radiotherapy to provide therapeutic benefit in NSCLC.

Suggested Reviewers:

Opposed Reviewers:

NYU
SCHOOL OF
MEDICINE

Mary Helen Barcellos-Hoff, Ph.D.
Professor of Radiation Oncology
Director of Radiation Biology
mhbarcellos-hoff@nyumc.org

September 15, 2014

Dr. Anthony L. Zietman, MD, FASTRO
Editor in Chief
International Journal of Radiation Oncology, Biology and Physics

Dear Dr. Zietman,

Thank you for the valuable comments and insights from the review of our original submission entitled "Attenuation of the DNA Damage Response by TGFβ Inhibitors Enhances Radiation Sensitivity of NSCLC Cells In Vitro and In Vivo". I am pleased to submit on the behalf of my co-authors our revised manuscript that addresses this critique of these preclinical therapeutic studies, which I hope you and the reviewers will deem appropriate for publication. I have included below a point-by-point response to the reviewers' comments indicated in blue

To recap, these studies were based on strong mechanism-based hypothesis that TGFβ inhibition can radiosensitize cancer cells based on our prior research showing its inhibition compromises ATM kinase activity. In this study we show that ATM activity is abrogated small molecule inhibition of the TGFβ type I receptor kinase and indeed radiosensitizes some human non-small cell lung cancer cell lines are as measured by clonogenic survival due to unrepaired DNA damage measured by comet assay. The administration of TGFβ neutralizing antibodies in the syngeneic mouse model of Lewis lung cancer enhances the efficacy of fractionated radiation therapy. These data open a new venue for the use of TGFβ inhibitors that are in clinical trials and show promise for efficacy in cancer therapy.

Sincerely,

Mary Helen Barcellos-Hoff, Ph.D.
Director of Radiation Biology
Departments of Radiation Oncology and Cell Biology

Reviewer #1: In this manuscript the authors describe the impact of TGF beta inhibition on radiosensitivity of NSCLC cell lines. The authors use both in vitro and in vivo experiments to demonstrate the effect of TGF beta inhibition.

While the manuscript has merit, there are a number of issues that should be addressed.

In figure 1B, the western blots have no loading control.

Loading control has been added to Figure 1A.

Figure 1C should be shown as a growth curve over 96 hours to demonstrate that both TGF beta and the inhibitor impact on growth. It is unclear what Figure 1A is demonstrating.

Regarding Figure 1C growth curve, the exponential growth phase of these cell lines is between 24-96 hrs. These experiments were conducted with our standard protocol (Cancer Research 2011, Clinical Cancer Research, 2010) for exponential growth phase; the cell density at 96 hr is too high for accurate determination of growth.

If it is that the H292 cells produce TGF beta on their own, then why are they most sensitive to exogenous TGF beta treatment in terms of growth as shown in Figure 1C. Also, should demonstrate production of TGF beta in all cell lines, if this is meaningful to outcomes.

Mutational inactivation of the TGFβ signaling pathway is a key event during NSCLC progression; functionally, some NSCLC tumor cells whose growth can no longer be inhibited by TGF-β become more aggressive and invasive. Nonetheless, we wanted to convey that some NSCLC cell lines both maintain TGFβ secretion and response to TGFβ. However, given the reviewers comment and the word limit, the data on TGFβ production has been removed.

Also, what does the term SMI in Figure 1C refer to.

SMI refer to “small molecular inhibitor”, and has been added in Figure 1C legend.

In Figure 2. If you posit that depletion of TGFbeta signalling is impacting on DNA damage response, then why is there no change in ATM -1981 serine phosphorylation in H292 cells if they exhibit decreased p-SMAD (as shown in Figure 1). This mechanism needs to be clarified.

There may be several routes by which TGFβ inhibition compromises DDR and multiple ways in which cancer cells evade TGFβ control. Cancer cells are highly heterogeneous in terms of genomic alterations that include sequence changes and epigenetics. The H292 appear to have lost a intermediary between Smad and ATM. We are currently investigating what this intermediary is.

Our hypothesis that TGFβ signaling is required for ATM kinase activity is well-supported by data in non-malignant human cells showing that TGFβ inhibition kinase exhibit decreases phosphorylation of ATM and its downstream DDR

targets p53, Chk2 and Rad17 (Cancer Research 2006), as well as attenuating DDR in the mammary epithelium in situ (Cancer Research 2002). We went on to demonstrate that TGFβ inhibition prior to radiation attenuates DNA damage recognition and enhances clonogenic cell killing in vitro and vivo in a panel of breast cancer and glioblastoma cells lines (Clinical Cancer Research 2010, Cancer Research 2011).

Notably, a new publication shows that Smad7 is important in ATM activity (Park, S., et al Smad7 enhances ATM activity by facilitating the interaction between ATM and Mre11-Rad50-Nbs1 complex in DNA double-strand break repair. Cell Mol Life Sci, 1-14, 2014.) Smad7 is downstream of Smad2/3. This has been elaborated in the discussion, e.g. "Nevertheless, the exact mechanism or pathways involving this TGFβ inhibition, which attenuates IR induced DNA damage responses has not been well described"(first paragraph, page 13).

Further, gamma h2AX foci for H292 should be shown and the experiment should be repeated in A547 cells.

We quantified foci and added the results of A549 and NCI-H292 cells as suggested. This has been provided in Figure 2B.

Also there should be quanitification of gamma H2AX foci as a time course experiment over a longer period until full resolution of the foci.

The objective here in assessing γH2AX, as previously reported, was to determine the molecular recognition of DNA damage as evidenced by phosphorylation of H2AX rather than repair/recovery kinetics.

Why were only ATM and p53 exxamined as markers of DDR? There are other proteins activated in the DDR pathway.

We used these two endpoints to evaluate whether NSCLC cell lines behave as shown in our prior studies in breast cancer and GBM in which TGFβ inhibition enhances radiation sensitivity and impairs DDR in support of translational potential. The detailed mechanism by which TGFβ affects DDR is the topic of other studies.

In Figure 3, this experiment should be repeated in the other cell lines (especially H292). I would also like to see the impact of endogenous TGF beta on the resolution of comet moments as this should produce the opposite effect.

The comet data were conducted only in LLC as a prelude to conducting in vivo experiments.

We have not conducted experiments in which TGFβ is added to cultures because the complex biology of TGFβ often results in non-linear dose dependence and even opposite responses. For instance, it has been recently published that both TGFβ inhibition and addition reduces cell migration (see "Concentration-dependent effects of transforming growth factor β1 on corneal wound healing" Wang, et al. Mol Vis. 2011;17:2835-46. PMID: 22128231). Because of this well-

known aspect of TGFβ biology, and since its addition is not clinically viable, we decided to not use this strategy in the studies reported in this manuscript

In figure 4, there needs to be statistics done on the clonogenic assays. The antibody 1D11 in LLC curve appears to overlap with no pretreatment. The DER10 in Figure F does not add to the story and should be removed.

We mistakenly omitted a statistical comparison in our original manuscript and have included it in our revision using ANOVA with Tukey test. As suggested by the reviewer and because of words limitation, we removed Figure 4F.

Again exogenous TGF beta should be added to the media and clonogenic assay repeated.

See response about adding TGFβ above.

There should be quanitification and statistics in Figure 5A. The delayed growth as posited by the authors is difficult to appreciate in the manner in which the data is presented in Figure 1C.

As suggested by the reviewer we added the quantification and statistics in Figure 5B. As shown in Figure 1C, NCI-H1299, A549 and LLC cells were refractory to TGFβ-mediated growth regulation, only H292 cells were growth-delayed by TGFβ.

Tumor mass at time of harvest should be quantified and noted.

Tumor mass at time of collection was quantified and included (Figure 5E).

Also, if histology could be examined for p-SMAD, that would greatly increase the value of the manuscript.

Data showing that radiation induces p-SMAD consistent with TGFβ activation and is blocked by 1D11 are part of another study and are currently being prepared for an upcoming publication. In the manuscript submitted here, we refer to this observation as unpublished data.

The introduction is confusing. Initially the authors discuss NSCLC and then abruptly shift to GBM and breast cancer.

In the introduction, we provided data and references relative to our same area of interest - TGFβ signaling and DDR- applied to different types of cancer, including breast and brain cancers. We aimed at providing the reader with sufficient background information about the topic that we have investigated in NSCLC. However, upon the reviewr suggestion, introduction has been edited for better understanding.

Please spell out TME in introduction.

This oversight is corrected.

The reference of tgf beta inhibitors in clinical trials needs to be expanded to underscore the importance of the current work.

This is an excellent point. The introduction now includes a summary of clinical trials involving TGFβ inhibition in combination with radiotherapy in patients with breast cancer and GBM (ClinicalTrials.gov identifiers: NCT01401062, NCT01220271).

The methods regarding the in vivo experiment needs clarification. How many injects of control or 1D11 antibodies were administered.

Regarding control or 1D11 antibodies injection"…receive 1D11 or 13C4 control antibody (10 mg/kg, intraperitoneal injection) 24 hr before localized irradiation and kept injecting every other days to termination"

How were the measurements made after two fractions, if there was such significant volume change, how is this accounted for in the growth curves?

"The measurements made after two fractions" means we measured the tumor after applying two fractions of 6Gy, i.e. the third day of irradiation. As shown in Figure 5F, we found that tumors in IR + 13C4 group presented similar tumor volume increase (compared to volume before IR) which indicated tumor growth has comparable kinetic with the two groups of sham irradiated tumors at this time point. However, the volume of half of the tumors (6/10) in IR+1D11 group decreased significantly.

Given that the experiments occurred when tumors reached 60-80 mm3 there is large margin for error on tumor measurements.

The mice were randomized when tumors reached 60-80 mm3, one day before treatment initiation, to create groups of similar comparable average volume. Each tumor was measured twice at each time-point during the experiment by two investigators to limit errors due to initial tumor small size and to increase accuracy. Add groups stats?

Reviewer #2: The authors of this study describe a study examining the effect of TGF-beta inhibition on radiosensitization of NSCLC cells using a small molecule TGBRI inhibitor and antibody against TGF-beta. The authors show data using pharmacologic experiments to suggest that TGF-beta signaling is critical for radiation response of NSCLC cells. Overall, I think this study is promising, but is somewhat derivative from work already performed with the same inhibitors in other cancers and in my mind has some deficiencies as detailed below. Finally, I would appreciate the consideration of addressing some other major and minor suggested revisions as below.

Major Revisions:

(1) How as growth inhibition measured in Figure 1C. There is no mention of the technique in the M&M, Results or Legend.
We described it in the M&M "Cell culture" (page 5 first paragraph).

(2) Why did the authors also not examine A549 cells in Figure 2A?
As suggested by the reviewer, we added the results of A549 cells.

(3) What about A549 and NCI-H292 cells for gamma-H2AX foci in Figure 2?
The data of A549 and NCI-H292 have been included in Figure 2B.

(4) At least one more cell line for the comet assay and preferably all of them would help confirm this interesting finding of increased DNA damage in LY364947 treated cells with IR (in Figure 3).
See response for Reviewer 1, questions (3) above as well concerning the repeating cells using comet assay. (I would restate the answer here. Also questions from Reviewer 1 are not numbered).

(5) The difference in gamma-H2AX foci between 13C4 +IR and ID11+IR treated animals is difficult to appreciate. Quantification with appropriate statistical comparisons would solidify the conclusion by the authors that there is difference
As shown in Figure 5A, γH2AX foci in vivo tissue is difficult to quantify by counting the number of foci. Here we quantify the mean intensity of γH2AX inside the nuclear region of interest. Notably, the effect of IR and TGFβ inhibition on the mean intensity is consistent with the in vitro results (Figure 5B)

(6) Changes in proliferation (Ki-67 IHC) or apoptosis (cleaved caspase 3 IHC or similar) can be examined in the tumor xenograft studies to help refine the mechanism(s) of tumor growth delay observed in the mouse studies.
The tumors were collected 1 hr and 2 weeks after completion of IR. Neither are appropriate time points to evaluate proliferation or apoptosis. We contend that the in vitro clonogenic survival data showing that TGFβ inhibition synergizes with RT is the basis for the increase in tumor growth delay.

(7) The statistical test used to differentiate the tumor growth delay in Figure 5B and 5D is marked as ANOVA in the accompanying Legend. ANOVA is useful to compare the means of three or more unmatched groups. The depiction in Figure 5B/5D suggests a comparison of only two groups (and it appears to be over time in 5B). ANOVA is not the correct test for this comparison.
We replaced ANOVA with unpaired two-tailed Student's t-test.
Tumor volume in exceeded size limitation defined by the Institution animal protocols the two sham-IR groups so we were required to terminate these two groups one day earlier.

(8) Page 13, 2nd paragraph is confusing and appears to be incorrect as written. There are multiple oncogenic driver sub-types most prominently in the adenocarcinoma histologic subtype. Of these oncogene driver subtypes, there is more than just EML4-ALK that has been shown to be targetable with agents that target specific oncoproteins, i.e. EGFR mutant subtype, ROS1-fusion subtype, RET-fusion subtypes, etc. Many of these have pre-clinical data and are in early phase clinical testing, but mutant EGFR oncoprotein targeting with erlotinib has been FDA approved even before crizotinib. We wished to underscore that anti-tumor agents often benefit only some NSCLC patients, due to tumor diversity

even with same histologic subtype. As we found NCI-H292 cells did not benefit from TGFβ inhibition, it is crucial for future clinical translation and to target a larger component of NSLC patients, to identify the explicit molecular biomarkers that will indicate the potential benefits from TGFβ inhibition in the context of RT.

(9) The references cited in this paragraph even given the Red J space constraints are not the best review articles that could be chosen (if possible the original literature would be ideal).
The reference 18 has been replaced by the original literature.

(10) Page 14, 1st paragraph is also a jumble of factoids that I am not sure really apply well to radiosensitizing strategies in NSCLC. Mitomycin-C is not a drug commonly used in lung cancer. Some of the other statements in this paragraph are without references and not correct. Would help if a lung cancer clinician read and helped edit this paragraph to express what the authors wanted to convey.
In this paragraph we aimed at briefly summarizing some of the approaches used to improve NSCLC radiation therapy, which mostly involve the use of a higher radiation dose or effective radiation sensitization. Chemotherapeutic reagents were most commonly used as radiosensitizers to synergize with RT in clinical settings, however these reagents do not specifically target tumor cells, and they increase radiation toxicity to normal tissues. Because of these intrinsic limitations of chemotherapeutic reagents, we postulated that TGFβ inhibitors selectively sensitize tumor response to radiation and at the same time protect normal lung from RT-induced fibrosis. In such clinical settings one could use selective escalation of the effective biologic IR dose to the tumor, and therefore improve local tumor control without increasing morbidity.

We are aware that Mitomycin-C is not used as first line in NSCLC, however it can be applied in NSCLC combination chemotherapy with concurrent radiotherapy (J Clin Oncol, 2010;28:3299-3306). In order not to misleading the reader we edited this paragraph and related references.

Minor Revisions:

(1) "TME" is never defined in text. Please define tumor microenvironment once before using "TME"
We have corrected this oversight.

(2) Why did the authors not check the TGFB levels of A549 and LLC?
Due to the reviewer 1's suggestion and the Journal word limit, we removed TGFB levels.

(3) Figure 1C, what is "SMI". Please define at least once.
We have corrected this oversight.

(4) Figure 2A - LLC panel is misaligned.
We have corrected this oversight.

***Detailed Response to Reviewers (No Author Details)**

Reviewer #1: In this manuscript the authors describe the impact of TGF beta inhibition on radiosensitivity of NSCLC cell lines. The authors use both in vitro and in vivo experiments to demonstrate the effect of TGF beta inhibition.

While the manuscript has merit, there are a number of issues that should be addressed.

In figure 1B, the western blots have no loading control.

Loading control has been added to Figure 1A.

Figure 1C should be shown as a growth curve over 96 hours to demonstrate that both TGF beta and the inhibitor impact on growth. It is unclear what Figure 1A is demonstrating.

Regarding Figure 1C growth curve, the exponential growth phase of these cell lines is between 24-96 hrs. These experiments were conducted with our standard protocol (Cancer Research 2011, Clinical Cancer Research, 2010) for exponential growth phase; the cell density at 96 hr is too high for accurate determination of growth.

If it is that the H292 cells produce TGF beta on their own, then why are they most sensitive to exogenous TGF beta treatment in terms of growth as shown in Figure 1C. Also, should demonstrate production of TGF beta in all cell lines, if this is meaningful to outcomes.

Mutational inactivation of the TGFβ signaling pathway is a key event during NSCLC progression; functionally, some NSCLC tumor cells whose growth can no longer be inhibited by TGF-β become more aggressive and invasive. Nonetheless, we wanted to convey that some NSCLC cell lines both maintain TGFβ secretion and response to TGFβ. However, given the reviewers comment and the word limit, the data on TGFβ production has been removed.

Also, what does the term SMI in Figure 1C refer to.

SMI refer to “small molecular inhibitor”, and has been added in Figure 1C legend.

In Figure 2. If you posit that depletion of TGFbeta signalling is impacting on DNA damage response, then why is there no change in ATM -1981 serine phosphorylation in H292 cells if they exhibit decreased p-SMAD (as shown in Figure 1). This mechanism needs to be clarified.

Our data in non-malignant cells show that TGFβ inhibition kinase exhibit decreased phosphorylation of ATM and downstream DDR markers p53, Chk2 and Rad17 (Cancer Research 2006). We have demonstrated that TGFβ inhibition prior to radiation attenuates DNA damage recognition and enhances clonogenic cell killing in vitro and vivo in a panel of cells lines such as mammary epithelium, breast cancer and glioblastomas (Cancer Research 2002, Clinical Cancer Research 2010, Cancer Research 2011). How this varies across cancer cell lines is an interesting question that we are actively pursuing. One avenue that we and others are investigating is whether TGFβ modulation of DDR in

cancer cells occurs through SMAD-dependent or SMAD-independent pathways. A recent publication shows that Smad7 is important (Park, S., et al (2014). Smad7 enhances ATM activity by facilitating the interaction between ATM and Mre11-Rad50-Nbs1 complex in DNA double-strand break repair. Cell Mol Life Sci, 1-14). Thus there may be several routes by which TGFβ inhibition compromises DDR. This has been elaborated in the discussion, e.g. "Nevertheless, the exact mechanism or pathways involving this TGFβ inhibition, which attenuates IR induced DNA damage responses has not been well described"(first paragraph, page 13).

Further, gamma h2AX foci for H292 should be shown and the experiment should be repeated in A547 cells.

We quantified foci and added the results of A549 and NCI-H292 cells as suggested. This has been provided in Figure 2B.

Also there should be quanitification of gamma H2AX foci as a time course experiment over a longer period until full resolution of the foci.

It is known that γH2AX foci reflect the presence of a DSB. Our objective here in assessing γH2AX, as previously reported, is to determine the molecular recognition of DNA damage. As suggested from the reviewer, it is excellent to quantify the γH2AX foci to know the effect of TGFβ inhibition on the DNA damage repair progression after RT. We is concerned that radiation induced γH2AX foci doesn't equate with DSB all situations which is dose and time dependence (Mutat Res. 2010, 704: 78-87). For example, normal human fibroblasts irradiated showed persistent foci for 5 days following 4 Gy of X-rays even though by that time all DSB should be fully resolved which supported the presence of γH2AX foci may not always signify the presence of a physical break.

Why were only ATM and p53 exxamined as markers of DDR? There are other proteins activated in the DDR pathway.

We used these two endpoints to evaluate whether NSCLC cell lines behave as shown in our prior studies in breast cancer and GBM in which TGFβ inhibition enhances radiation sensitivity and impairs DDR as motivation for translational research rather than mechanism, which is the topic of other studies.

In Figure 3, this experiment should be repeated in the other cell lines (especially H292). I would also like to see the impact of endogenous TGF beta on the resolution of comet moments as this should produce the opposite effect.

Because of manuscript size restrictions, we reported data that further confirmed prior publications in breast cancer and GBM. We believe that the comet assay in LLC cell lines may support the translational potential that then applied in the animal experiments.

In figure 4, there needs to be statistics done on the clonogenic assays. The antibody 1D11 in LLC curve appears to overlap with no pretreatment. The DER10 in Figure F does not add to the story and should be removed.

We mistakenly omitted a statistical comparison in our original manuscript and have included it in our revision using ANOVA with Tukey test. As suggested by the reviewer and because of words limitation, we removed Figure 4F.

Again exogenous TGF beta should be added to the media and clonogenic assay repeated.

We appreciate the complex biology of TGFβ that might result in non-linear dose dependence and even opposite responses. For instance, it has been recently published that both TGFβ inhibition and addition reduces cell migration (see "Concentration-dependent effects of transforming growth factor β1 on corneal wound healing. Wang, et al. Mol Vis. 2011;17:2835-46. PMID: 22128231"), . Because of this well-known aspect of TGFβ biology and since its addition is not clinically viable, we decided to not use this strategy in the studies reported in this manuscript

There should be quanitification and statistics in Figure 5A. The delayed growth as posited by the authors is difficult to appreciate in the manner in which the data is presented in Figure 1C.

As suggested by the reviewer we added the quantification and statistics in Figure 5B. As shown in Figure 1C, NCI-H1299, A549 and LLC cells were refractory to TGFβ-mediated growth regulation, only H292 cells were growth-delayed by TGFβ.

Tumor mass at time of harvest should be quantified and noted.

Tumor mass at time of collection was quantified and included (Figure 5E).

Also, if histology could be examined for p-SMAD, that would greatly increase the value of the manuscript.

Data showing that radiation induces p-SMAD consistent with TGFβ activation and is blocked by 1D11 are part of another study and are currently being prepared for an upcoming publication. In the manuscript submitted here, we refer to this observation as unpublished data.

The introduction is confusing. Initially the authors discuss NSCLC and then abruptly shift to GBM and breast cancer.

In the introduction, we provided data and references relative to our same area of interest - TGFβ signaling and DDR- applied to different types of cancer, including breast and brain cancers. We aimed at providing the reader with sufficient background information about the topic that we have investigated in NSCLC. However, upon the reviewr suggestion, introduction has been edited for better understanding.

Please spell out TME in introduction.

This oversight is corrected.

The reference of tgf beta inhibitors in clinical trials needs to be expanded to underscore the importance of the current work.

This is an excellent point. The introduction now includes a summary of clinical trials involving TGFβ inhibition in combination with radiotherapy in patients with breast cancer and GBM (ClinicalTrials.gov identifiers: NCT01401062, NCT01220271).

The methods regarding the in vivo experiment needs clarification. How many injects of control or 1D11 antibodies were administered.

Regarding control or 1D11 antibodies injection"…receive 1D11 or 13C4 control antibody (10 mg/kg, intraperitoneal injection) 24 hr before localized irradiation and kept injecting every other days to termination"

How were the measurements made after two fractions, if there was such significant volume change, how is this accounted for in the growth curves?

"The measurements made after two fractions" means we measured the tumor after applying two fractions of 6Gy IR,at the third day of irradiation. As shown in Figure 5F, we found that tumorsin IR + 13C4 group presented similar tumor volume increase (compared to volume before IR) which indicated tumor growth has comparable kinetic with the two groups of sham irradiation tumors at this time point. However, the volume of half of the tumors (6/10) in IR+1D11 group decreased significantly.

Given that the experiments occurred when tumors reached 60-80 mm3 there is large margin for error on tumor measurements.

The mice were randomized when tumors reached 60-80 mm3, one day before treatment initiation, to create groups of similar comparable average volume. Each tumor was measured twice at each time-point during the experiment by two investigators to limit errors due to initial tumor small size and to increase accuracy.

Reviewer #2: The authors of this study describe a study examining the effect of TGF-beta inhibition on radiosensitization of NSCLC cells using a small molecule TGBRI inhibitor and antibody against TGF-beta. The authors show data using pharmacologic experiments to suggest that TGF-beta signaling is critical for radiation response of NSCLC cells. Overall, I think this study is promising, but is somewhat derivative from work already performed with the same inhibitors in other cancers and in my mind has some deficiencies as detailed below. Finally, I would appreciate the consideration of addressing some other major and minor suggested revisions as below.

Major Revisions:

(1) How as growth inhibition measured in Figure 1C. There is no mention of the technique in the M&M, Results or Legend.
We described it in the M&M "Cell culture" (page 5 first paragraph).

(2) Why did the authors also not examine A549 cells in Figure 2A?
As suggested by the reviewer, we added the results of A549 cells.

(3) What about A549 and NCI-H292 cells for gamma-H2AX foci in Figure 2?
The data of A549 and NCI-H292 have been included in Figure 2B.

(4) At least one more cell line for the comet assay and preferably all of them would help confirm this interesting finding of increased DNA damage in LY364947 treated cells with IR (in Figure 3).
See response for Reviewer 1, questions (3) above as well concerning the repeating cells using comet assay. (I would restate the answer here. Also questions from Reviewer 1 are not numbered).

(5) The difference in gamma-H2AX foci between 13C4 +IR and ID11+IR treated animals is difficult to appreciate. Quantification with appropriate statistical comparisons would solidify the conclusion by the authors that there is difference
As shown in Figure 5A, γH2AX foci in vivo tissue is difficult to quantify by counting the number of foci. Here we quantify the mean intensity of γH2AX inside the nuclear region of interest. Notably, the effect of IR and TGFβ inhibition on the mean intensity is consistent with the in vitro results (Figure 5B)

(6) Changes in proliferation (Ki-67 IHC) or apoptosis (cleaved caspase 3 IHC or similar) can be examined in the tumor xenograft studies to help refine the mechanism(s) of tumor growth delay observed in the mouse studies.
The tumors were collected 1 hr and 2 weeks after completion of IR. Neither are appropriate time points to evaluate proliferation or apoptosis. We contend that the in vitro clonogenic survival data showing that TGFβ inhibition synergizes with RT is the basis for the increase in tumor growth delay.

(7) The statistical test used to differentiate the tumor growth delay in Figure 5B and 5D is marked as ANOVA in the accompanying Legend. ANOVA is useful to compare the means of three or more unmatched groups. The depiction in Figure 5B/5D suggests a comparison of only two groups (and it appears to be over time in 5B). ANOVA is not the correct test for this comparison.
We replaced ANOVA with unpaired two-tailed Student's t-test.
Tumor volume in the two sham-IR groups exceeded size limitation defined by the Institution animal protocols so we were required to terminate these two groups one day earlier.

(8) Page 13, 2nd paragraph is confusing and appears to be incorrect as written. There are multiple oncogenic driver sub-types most prominently in the adenocarcinoma histologic subtype. Of these oncogene driver subtypes, there is more than just EML4-ALK that has been shown to be targetable with agents that target specific oncoproteins, i.e. EGFR mutant subtype, ROS1-fusion subtype, RET-fusion subtypes, etc. Many of these have pre-clinical data and are in early phase clinical testing, but mutant EGFR oncoprotein targeting with erlotinib has been FDA approved even before crizotinib. The references cited in this paragraph even given the Red J space constraints are not the best review articles that could be chosen (if possible the original literature would be ideal).

We wished to underscore that anti-tumor agents often benefit only some NSCLC patients, due to tumor diversity even with same histologic subtype. As we found NCI-H292 cells did not benefit from TGFβ inhibition, it is crucial for future clinical translation and to target a larger component of NSLC patients, to identify the explicit molecular biomarkers that will indicate the potential benefits from TGFβ inhibition in the context of RT.
The reference 18 has been replaced by the original literature.

(9) Page 14, 1st paragraph is also a jumble of factoids that I am not sure really apply well to radiosensitizing strategies in NSCLC. Mitomycin-C is not a drug commonly used in lung cancer. Some of the other statements in this paragraph are without references and not correct. Would help if a lung cancer clinician read and helped edit this paragraph to express what the authors wanted to convey.
In this paragraph we aimed at briefly summarizing some of the approaches used to improve NSCLC radiation therapy, which mostly involve the use of a higher radiation dose or effective radiation sensitization. Chemotherapeutic reagents were most commonly used as radiosensitizers to synergize with RT in clinical settings, however these reagents do not specifically target tumor cells, and they increase radiation toxicity to normal tissues. Because of these intrinsic limitations of chemotherapeutic reagents, we postulated that TGFβ inhibitors selectively sensitize tumor response to radiation and at the same time protect normal lung from RT-induced fibrosis. In such clinical settings one could use selective escalation of the effective biologic IR dose to the tumor, and therefore improve local tumor control without increasing morbidity.

We are aware that Mitomycin-C is not used as first line in NSCLC, however it can be applied in NSCLC combination chemotherapy with concurrent radiotherapy (J Clin Oncol, 2010;28:3299-3306). In order not to misleading the reader we edited this paragraph and related references.

Minor Revisions:

(1) "TME" is never defined in text. Please define tumor microenvironment once before using "TME"
We have corrected that

(2) Why did the authors not check the TGFB levels of A549 and LLC?
Due to the reviewer 1's suggestion and the Journal word limit, we removed TGFB levels.

(3) Figure 1C, what is "SMI". Please define at least once.
We have corrected this oversight.

(4) Figure 2A - LLC panel is misaligned.
We have corrected this oversight.

*Title Page (WITH Author Details)

Attenuation of the DNA Damage Response by TGFβ Inhibitors Enhances Radiation Sensitivity of NSCLC Cells *In Vitro* and *In Vivo*

Shisuo Du, MD, PhD, [1] Sophie Bouquet, PhD, [1] Chen-Hao Lo, MS [1], Ilenia Pellicciotta, PhD, [1] Shiva Bolourchi, PhD, [1] Renate Parry, PhD,[2] and Mary Helen Barcellos-Hoff, PhD[1,3]

[1] Department of Radiation Oncology, New York University School of Medicine, 566 First Avenue, New York, NY 10016, USA

[2] Varian Medical Systems, Inc., Palo Alto CA

[3] Address reprint requests to

Mary Helen Barcellos-Hoff

Department of Radiation Oncology

New York University, School of Medicine

566 First Avenue, New York, NY10016

Phone: 212-263-3021

E-mail: mhbarcellos-hoff@nyumc.org

Running title: TGFβ Inhibition in non small cell lung cancer (NSCLC) RT

Conflicts of Interest: Dr. Barcellos-Hoff reports grants from Varian Medical Systems, Inc., and non-financial support from Genzyme, during the conduct of the study;

Dr. Sophie finished her postdoc at NYU (and so the experiments in this paper) and went to work for Roche where she's still an employee.

Funding Acknowledgment: This research was supported by Varian Medical Systems, Inc.

Thanks to Dr. Raheleh Hatami for having edited the manuscript.

Attenuation of the DNA Damage Response by TGFβ Inhibitors Enhances Radiation Sensitivity of NSCLC Cells *In Vitro* and *In Vivo*

Summary

Our study shows that TGFβ inhibition synergizes with RT through control of the DNA damage response, which leads to greater tumor cell kill. There are several TGFβ inhibitors in clinical trials. The excellent safety profiles demonstrated in these clinical trials, as well as the possibility of protection from late complications of lung fibrosis, provide further motivation for assessing TGFβ inhibitors as an adjunct to RT for NSCLC.

Attenuation of the DNA Damage Response by TGFβ Inhibitors Enhances Radiation Sensitivity of NSCLC Cells *In Vitro* and *In Vivo*

Abstract

Purpose To determine whether inhibition of TGFβ increases the response to radiotherapy in human and mouse NSCLC cancer cells *in vitro* and *in vivo*.

Methods TGFβ-mediated growth response and pathway activation were examined in human, NCI-H1299, NCI-H292, and A549 NSCLC cell lines and murine Lewis lung cancer (LLC) cells. Cells were treated *in vitro* with LY364947, a small molecule inhibitor of the TGFβ type I receptor kinase, prior to determination of clonogenic survival following graded radiation doses. DNA damage recognition was measured by γH2AX and repair was assessed using a comet assay in irradiated cells pretreated with LY364947. Levels of protein involved in the DNA damage response were measured using Western blotting. Mice bearing LLC syngeneic murine subcutaneous tumors were treated with a pan-isoform TGFβ neutralizing monoclonal antibody, 1D11, and fractionated radiation therapy.

Results Irrespective of sensitivity to TGFβ growth regulation, 3 of the 4 NSCLC cell lines pretreated with LY364947 prior to radiation exposure exhibited compromised DNA damage ~~recognitions~~ response. This was ~~as~~ indicated by decreased ATM and p53 phosphorylation, reduced γH2AX foci, and increased radiosensitivity, which was ~~as~~ measured by clonogenic survival. TGFβ signaling inhibition in irradiated LLC cells resulted in greater residual DNA damage. Mice bearing LLC subcutaneous tumors treated with 1D11 also exhibited fewer γH2AX

foci shortly after irradiation and greater tumor growth delay in combination with fractionated radiation exposure.

Conclusions These results indicate that TGFβ inhibition prior to radiation attenuates DNA damage recognition, enhances clonogenic cell killing and promotes tumor growth delay. These results , ~~which~~ suggest that concurrent TGFβ inhibition and radiotherapy will provide therapeutic benefit in NSCLC.

Key Words: ionizing radiation; non-small cell lung cancer; TGFβ; small molecule inhibitor; neutralizing antibody

Introduction

Improved treatment of non-small-cell lung cancer (NSCLC), which is one of the leading causes of deaths from cancer worldwide, is urgently needed [1]. Approximately 70% of NSCLC patients receive radiotherapy (RT), either alone or in combination with other treatment modalities such as surgery or chemotherapy. Biologically augmenting tumor cell radiosensitivity can play a crucial role~~s~~ in determining treatment success [2]. TGFβ ligands are enriched in the tumor microenvironment (TME), and ~~where~~ their production by stromal or tumor cells varies~~y~~ according to the tumor phenotype. In NSCLC, increased TGFβ activity correlates with tumor progression and increased tumor angiogenesis [3,4]. TGFβ signaling activation in ~~tTumor microenvironment~~the ~~(~~TME~~)~~ has been identified as a key factor for chemotherapy resistance in NSCLC [4], in addition to its well-recognized impact on tumor promoting effects in progression and metastasis. Currently, TGFβ inhibitors are in clinical trials for several types of cancer including ~~breast cancer and~~ glioblastoma (GBM) and breast cancer (reviewed in [5]). TGFβ signaling blockade augments ~~glioblastoma (GBM)~~ response to chemoradiation in GBM preclinical models [6,7], and specifically inhibits GBM cancer stem cell renewal after radiation [8]. TGFβ inhibition promotes clonogenic cell death in irradiated GBM and both mouse and human breast cancer cell lines ~~and GBM cells~~ *in vitro*~~,~~ ~~S~~and ~~s~~ystemically neutralizing TGFβ enhances RT actions in GBM and breast cancer preclinical models [6,8,9].

Growing evidence supports a specific role for TGFβ in mediating the rapid execution of the DNA damage response (DDR) [10]. *Tgfb1* null keratinocytes irradiated *in vitro* exhibit reduced ataxia telangiectasia mutated (ATM) protein auto-phosphorylation and kinase activity. ~~,~~ This ~~which in~~

~~turn~~then decreases recognition of the sites of DNA damage, which is evident~~ced~~ by decreased γH2AX foci and impaired~~s~~ critical DNA damage transducers Chk2, p53, and Rad17 that ~~in turn,~~ abrogate the appropriate cell fate decisions [11]. Notably, a TGFβ type I receptor kinase small molecule inhibitor phenocopies genetic depletion in human epithelial cells.

Little is known about the contribution of TGFβ to the tumor response to radiation in NSCLC. TGFβ activation is efficiently induced by ionizing radiation, in part due to the presence of a redox sensitive motif in the latency associated peptide (reviewed in [12]). Based on the efficacy in breast and brain tumors, it is plausible that TGFβ inhibition through compromising DDR after IR-induced DNA damage could improve radiosensitivity of NSCLC irrespective of cell growth sensitivity. TGFβ signaling inhibitors are generally safe and may be efficacious in several clinical applications. Clinical trials involving TGFβ inhibition in combination with radiotherapy in patients with breast cancer and –GBM –have progressed (ClinicalTrials.gov identifiers: NCT01401062, NCT01220271). ~~Using a small molecule inhibitor of the TGFβ type I receptor kinase and a pan-neutralizing TGFβ antibody currently in clinical development, W~~Hhere, w~~w~~e determined the role of TGFβ signaling in DDR following irradiation in NSCLC cell lines and murine Lewis lung carcinoma (LLC) tumors. We found that although most of human and murine NSCLC cell lines were refractory to TGFβ-mediated growth regulation, TGFβ inhibition increased radiosensitivity of NSCLC cells, compromised DDR, and the combination of TGFβ neutralizing antibody with fractionated RT significantly increased tumor growth control. These results suggest that concurrent TGFβ inhibition and radiotherapy will provide therapeutic benefit in clinical settings for NSCLC treatment.

Formatted: (Asian) Chinese (PRC)

Formatted: (Asian) Chinese (PRC)

Formatted: Font: 12 pt, (Asian) Chinese (PRC)

Formatted: Font: 11 pt

Formatted: Space After: 10 pt

Methods

Cell culture

Human NCI-H1299, NCI-H292, A549 and murine LLLC cells were purchased from American Type Culture Collection (Manassas, VA). LLC Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Gibco) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO). Human NCI-H1299, NCI-H292 and A549 were cultured in RPMI-1640 Medium with 10% FBS (Sigma-Aldrich, St. Louis, MO). Cells were treated in 10% serum replacement medium (SRM; Knockout SR, Life Technologies, Inc., Carlsbad, CA) containing either 500 pg/ml TGFβ (R&D Systems, Minneapolis, MN), 400 nM small molecule inhibitor of the TGFβ type I receptor kinase, LY364947 ([3-(Pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole; Lilly designation HTS466284; Cat# 616451, Calbiochem, St. Louis, MO) or 1D11, a pan-isoform, neutralizing TGFβ monoclonal antibody or 13C4, murine monoclonal isotype control antibody (kindly provided by Genzyme, Framingham, MA). For growth studies, cells were trypsinized and counted using a Coulter counter at 24-hour and 48-hour post treatment.

Clonogenic assay

To assess clonogenic survival of cells in monolayer culture, human and murine lung cancer cell lines were grown for 48 hours to about 70% confluence, at which point media was replaced with serum replacement media. Cells were treated with 400 nmol/L of LY364947 kinase inhibitor or 10 μg/mL pan-specific TGFβ-neutralizing antibody 1D11 or control antibody 13C4 for 48 h before and 3 h post radiation exposure. Cells were irradiated with graded doses of up to 8 Gy using a Varian Clinac 2300 C/D linear accelerator with 1cm bolus below (Varian, Palo

Alto, CA). Colony formation of 3 biological replicates was averaged for each treatment and corrected according to plating efficiency of respective controls. Sensitization dose enhancement ratios (DER10) were calculated as the ratio of doses required to achieve 10% surviving fraction for cells without and with TGFβ inhibition.

γH2AX foci

Tumor cryosections or cells grown on chamber slides were fixed using 2% paraformaldehyde for 20 minutes at room temperature followed by permeabilization with 100% methanol for 20 minutes at −20°C. Then specimens were blocked with the supernatant of 0.5% casein/PBS, stirred for 1 hour, and incubated with a mouse monoclonal antibody against γH2AX (clone JBW301, Upstate Biotechnology, NY) overnight at 4°C as previously described [9]. Specimens were imaged using a 40× objective with 0.95 numerical aperture Zeiss Plan-Apochromat objective on a Zeiss Axiovert (Zeiss) equipped with epifluorescence. All images were acquired with a CCD Hamamatsu Photonics monochrome camera at 1,392 × 1,040 pixel size, 12 bits per pixel depth using the Metamorph imaging platform (Molecular Devices, Inc.).

Comet assay

LLC cells were cultured and treated with 400 nmol/L of LY364947 kinase inhibitor as described above and irradiated with 5 Gy. LLC cells were dissociated 0.5 or 4 hours after irradiation for single-cell gel electrophoresis at 19 V (300mAM, 40 min) and analysis by neutral comet assay (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. SYBR green-stained DNA comets were imaged at 400x magnification and the extent of DNA breaks was quantified as tail moment using Comet Score software.

Western analysis

Cells were grown in complete media for 48 hour, treated with LY364947, irradiated with 5 Gy, and lysed after 1 hr. Alternatively, cells were treated with 500 pg/mL TGFβ and lysed after 30 minutes. Protein estimation was carried out using the BCA protein assay kit from Pierce. One hundred micrograms of protein was electrophoresed on a 4% to 15% gradient gel from BioRad and transblotted on polyvinylidene difluoride membrane. The immunoblots were incubated with one of the following primary antibodies at 1/500 dilution: Smad2 serine 465/467 phosphorylation (Cell Signaling, Danvers, MA), Smad2/3 (BD, Franklin Lakes, NJ), p53 serine 15 phosphorylation (Cell Signaling , Danvers, MA), p53 serine 20 phosphorylation (Cell Signaling, Danvers, MA), p53 (Neomarkers, Fremont, CA), ATM serine1981 phosporylation (Epitomics, Burlingame, CA), and ATM, clone 2C1 (GeneTex, Irvine, CA) and detected with infrared labeled antibodies using a LiCor Odyssey system.

Tumor studies

All animal experiments were carried out in accordance with guidelines specified by X X University's institutional animal care and use committee. Female C57BL/6 mice, age 6 to 8 weeks, obtained from Taconic were housed in a temperature-controlled animal care facility with a 12-hour light–dark cycle and allowed chow and water *ad libitum*. LLC cells (10^5) were injected into the right flank of mice and allowed to grow until the tumors reached an average size of 60-80 mm^3. Animals were distributed randomly into groups (n=5-10) to receive 1D11 or 13C4 control antibody (10 mg/kg, intraperitoneal injection) 24 hr before localized irradiation and ~~kept injecting~~were injected every other day~~s~~ to termination. Radiation was delivered at 600

cGy/min with 6MV X-rays with Varian Clinac 2300 C/D linear accelerator fitted with a 25-mm radiosurgery conical collimator (Varian, Palo Alto, CA). Superflab bolus (1.5-cm tissue equivalent material) was placed over the tumor.

~~**TGFβ1 assay**~~

~~Levels of total and active TGFβ1 in the supernatant of human H1299 and H292 tumor cells were assessed by a multiplex assay using a electrochemiluminescence-based ELISA (Mesoscale Discovery®). The serum-free media conditioned by cells was collected after 72 hr. To measure active TGFβ, the supernatant was activated by acid, as recommended by the manufacturer.~~

Results

Response of human and murine NSCLC cell lines to TGFβ

Mutational inactivation of the TGFβ signaling pathway in human NSCLC is associated with specific histological subtypes, more aggressive tumor behavior, and reduced patient survival [2]. NCI-H1299 and NCI-H292 are aggressive tumor cell lines established from a lymph node metastasis of a pulmonary carcinoma; A549 cell line is from a primary human adenocarcinoma; and LLC is a highly metastatic murine lung cancer cell line. ~~NCI-H1299 cells produced both 6.4 pg/mL active and 1503 pg/mL total TGFβ in serum-free conditions, while NCI-H292 produced 14.4 pg/mL and 231.4pg/mL respectively **(Figure 1A)**.~~ Since NSCLC cells can selectively evade TGFβ growth regulation while maintaining signaling, we next examined ~~whether~~ the TGFβ canonical pathway, indicated by Smad2 phosphorylation (p-Smad2). TGFβ treatment significantly induced p-Smad2, which was suppressed by TGFβ inhibitor LY364947 **(Figure ~~1B~~1A)**. These data indicate that receptor-mediated signaling in response to TGFβ is intact. Since cell proliferation status constitutes an important component of the tumor cell radiosensitivity, we tested NSCLC tumor sensitivity to TGFβ-mediated growth inhibition. Only H292 cells were growth-inhibited by TGFβ, whereas NCI-H1299, A549, and LLC cells were refractory to TGFβ-mediated growth regulation **(Figure ~~1C~~1B).**

Blocking TGFβ signaling attenuates DNA damage response in NSCLC cells *in vitro*

We next assessed the radiation-induced DNA damage response. LY364947 significantly decreased phosphorylation of ATM at serine 1981~~,~~ and p53 phosphorylation at serine 15 in

irradiated LLC cells (**Figure 2A)**. Although p53 was undetectable in NCI-H1299 cells carrying a *Tp53* homozygous deletion, phosphorylation of ATM Ser1981 was attenuated after LY364947 treatment. No effect of LY364937 was observed in NCI-H292 cells. As shown in our prior studies [8,9], radiation-induced γH2AX foci formation was markedly reduced by pre-treatment with LY364947 in both NCI-1299 and LLC cells *in vitro* (**Figure 2B~~, C~~**).

Next, w~~W~~e conducted neutral comet assays to investigate the effect of TGFβ inhibition on DNA damage induction and resolution at 30 min and 4 hrs respectively after LLC cells were irradiated with 5 Gy (**Figure 3A**). The initial comet tail moment was similar for irradiated cells with or without LY364947, indicating comparable initial DNA damage. Most radiation-induced DSB are repaired in the first 1-6 hr after RT [13]. The comet tail moment decreased compared to 30 min after 5Gy IR. In contrast, the comet~~ment~~ tail moment failed to decline in irradiated cells treated with LY364947 (**Figure 3B, C**). Thi~~s~~e persisten~~t~~ce ~~of~~ DNA damage indicates that treatment with LY364947 impairs DNA damage repair.

Inhibition of TGFβ signaling sensitizes NSCLC cell lines to irradiation

The clonogenic assay is a gold standard for radiosensitivity estimation. Human cell lines NCI-H1299 and A549 were radiosensitized by pre-treatment with LY364947, consistent with the DDR related proteins analysis, whereas radiosensitization was limited in NCI-H292 cells (**Figure 4 A-C**). ~~Similar~~ These results ~~as NCI-H292~~ were also detected ~~from~~ when TGFβ was inhibited~~ion~~ in NCI-H460, which is a large cell lung cancer carcinoma (data not shown). We next compared the small molecular inhibitor and a pan-isoform TGFβ neutralizing antibody, 1D11, using the LLC

cell line. Clonogenic survival showeds that murine LLC cells were comparably radiosensitized by pre-treatment with either 1D11 (**Figure 4D)** or LY364947 **(Figure 4E**). ~~The DER_{10} survival is between 1.1± 0.14 and 1.2± 0.06 based triplicate determinations (**Figure 4F)**.~~

Combination of TGFβ inhibitor with fractionated RT increases LLC tumor growth delay

TGFβ neutralizing antibodies currently in clinical development have demonstrated safety and efficacy in several studies [14,15]. To test the efficacy in combination with RT, we next examined LLC tumor bearing mice treated with 1D11 alone or in combination with fractionated RT. C57BL/6 mice bearing subcutaneous LLC tumors were treated with 5 daily fractions of 6 Gy and/or 1D11 or 13C4 (10mg/kg i.p.). Some tumors were randomly selected for harvesting 1 hr after irradiation to assess DDR by γH2AX foci staining. Similar to the *in vitro* data from LLC cells, γH2AX foci were reduced in irradiated tumors excised from hosts receiving treatment of 1D11 (**Figure 5A and B**). Notably, 1D11 alone had no significant effect on tumor growth compared with control ~~antibody,~~ 13C4 antibody ~~,~~ treated mice. However, when combined with RT, a tumor growth delay was considerably extended compared to mice receiving RT and 13C4 antibody (**Figure ~~5B~~ 5C and ~~C~~D**). Tumor volume at the experiment termination (day 27) was significantly smaller for mice treated with 1D11 and RT when compared to RT and 13C4 antibody. Interestingly, more than half (6/11) the mice showed tumor growth rate decrease after the first two fractions of 6Gy when treated with 1D11, in which the mean volume increment was 7.9±6.9 mm^3~~.~~ (tumor weight 0.74±0.10g). However, tumor growth in 13C4 antibody treated mice had the same growth rate as the non-irradiated tumors, with a mean volume increase of 34.8±9.7 mm^3 (tumor weight ~~,~~0.34±0.04g)~~.~~ (**Figure ~~5D~~5E and F**). These data

support the potential benefit of TGFβ inhibition in the context of RT, which attenuates the DDR, radiosensitizes tumor cells, and promotes tumor control *in vivo*.

Discussion

Our study demonstrates that pharmaceutical TGFβ inhibition compromises the DNA damage recognition~~,~~ ~~and~~ ~~thus~~ repair, and ~~and~~ ultimately~~,~~ tumor cell survival. The murine tumor cell line and most of the human NCSCL cells were radiosensitized independent of sensitivity to TGFβ-mediated growth inhibition. This ~~,~~ suggest~~sing~~ that this strategy would be effective across lung cancer subtypes. Additionally, this was further supported by our *in vivo* LLC syngeneic subcutaneous tumors, where tumor growth control was significantly improved by use of neutralizing antibodies concurrent with fractionated RT.

DNA is the main target for radiation-induced cell killing. Since there is considerable redundancy in the cell's ability to repair DNA damage, modulating the response to ionizing radiation through the inhibition of DNA repair has been a longstanding aim in translational RT research [16]. Previously, we demonstrated that TGFβ is involved in ATM activation in epithelial cells [11] and chemical or genetic inhibition of TGFβ signaling in these cells led to reduced ATM activation and increased tumor cell radiosensitivity in breast and brain tumor cell lines [8,9]. Consistent with our earlier studies, here we show that TGFβ inhibition prior to irradiation resulted in reduced phosphorylation of H2AX and p53 in cultured NSCLC cells. Moreover, LLC tumors in mice treated with TGFβ inhibitors prior to radiation exposure *in vivo* exhibited less γH2AX foci formation, a nuclear marker of the rapid molecular radiation response. Interestingly, 1D11

neutralizing antibodies showed immediate tumor growth inhibition after two fractions. At this time point post-IR, tumor cell death results mainly from aberrant repair of radiation-induced DNA damage. This is consistent with a direct effect on radiosensitvity due to compromising the DNA damage recognition. Here, NCI-H1299 cells, which carry ~~ing~~ a homozygous deletion of the p53 protein, were still sensitized by TGFβ inhibition. Nevertheless, the exact mechanism or pathways involving this TGFβ inhibition that~~,~~ ~~which~~ attenuat~~es~~es IR induced DNA damage responses has not been well described up until now. Furthermore, the limited published data only has suggested that the sensitizing effect of TGFβ inhibition is p53 independent.

Therapeutic resistance and RT induced lung damage are major challenges for NSCLC. This cancer ~~, which~~ consist~~s~~s of heterogeneous histologies, with the most common types being adenocarcinoma, large cell carcinoma, and squamous cell carcinoma. The differential response to anti-tumor treatments among these different histologies is a general problem in treating NSCLC [17]. Since most of NSCLC harbor mutations with different clinical characteristics, it is not surprising that till now only one drug, crizotinib used in~~in~~ EML4-ALK–positive patients, has proven to be particularly effective ~~in~~ for this specific subpopulation. This “tailored treatment” has resulted in an unprecedented survival benefit [18,19]. Here, we found that TGFβ inhibition had little effect on IR-induced DDR related proteins in NCI-H292, which were not radiosensitized. This suggests that that benefit will be restricted to certain tumors; h~~. H~~owever, further studies are critical in order to identify the molecular biomarkers that will indicate the potential benefits from inhibiting TGFβ ~~inhibition~~ in RT.

Hypofractionated stereotactic body radiation therapy (SBRT) allows for escalation of the fractional dose. ~~, which~~ This is important for~~to~~ not only improvin~~g~~e the local control rate of tumors, but ~~and~~ the overall survival for medically inoperable patients with early-stage NSCLC [20]. However, there are still many issues to be elucidated. Radiation dose and fractionation vary between institutions and protocols because there is no biologically effective dose guideline. Importantly, radiation-induced normal tissue damage is still the biggest obstacle, which limits the dose escalation of SBRT in NSCLC [~~21-23~~21,22]. Another approach with an enormous appeal involves the development of nontoxic, yet effective, molecularly targeted radiosensitizers. If a drug selectively sensitizes the tumor response to radiation, one could use selective escalation of the effective biological dose to the tumor, therefore, improving local tumor control without increasing morbidity. Drugs such as gemcitabine, cisplatin, and docetaxel ~~cisplatin and mitomycin C~~ have been demonstrated to radiosensitize tumors in NSCLC ~~and head and neck cancers~~ [23, 24]. ~~PI3K-AKT [12] and nuclear factor-κb (NF-κB)[25] are also regarded as the most well-studied survival pathways, which have a clear role in the response and sensitivity to RT.~~ Concurrently, these sensitizers increase the rates of local tumor control and in some cases overall survival [25]. As these radiosensitizers are not specific for tumor cells, they also increase radiation toxicity to normal tissues. This nonspecific mechanism of action is one of the major limiting factors of many radiation modulators. It is expected that chemotherapy-mediated inhibition of DNA repair mechanisms would synergize the TGFβ inhibition, as shown in combination with Temozolomide and IR in GBM preclinical models [6].

Formatted: Font: 12 pt, Check spelling and grammar

Formatted: Font: 12 pt, Check spelling and grammar

Unfortunately, radiation-induced lung injury is a major obstacle in NSCLC treatment. Since the lung represents the most RT sensitive tissue, this results in fatal complications. ~~-~~Among the many cytokines involved in this process, TGFβ is thought to play a pivotal role [26,27]. TGFβ is overexpressed at sites of injury, which contributes to delayed tissue fibrosis after radiation and chemotherapy. Various interventional therapies that aim to block TGFβ signaling or ~~reduce~~ decrease tissue levels of TGFβ have proven to be effective in reducing the development of fibrosis in the lung, liver, and intestines [26-28]. Reducing normal tissue injury by TGFβ inhibition is undoubtedly significant in RT dose-escalation for lung cancer and will increase tumor control probability. In conclusion, targeting the TGFβ pathway is a promising novel treatment candidate for NSCLC patients, as this s~~ is a promising candidate for NSCLC patients, as this will s~~electively increases tumor cell kill~~ing~~ and local tumor control, while potentially limiting ~~l~~late effects in the surrounding normal tissue. ~~-~~

Conclusions

Our data provides a strong preclinical rationale that TGFβ inhibitors have therapeutic benefit ~~from TGFβ inhibitors exploited~~ in the context of RT, via control of the DNA damage response by ~~that leads to greater~~increasing radiation sensitivity.

Bibliography

1. Kruse JJ, Floot BG, te Poele JA, Russell NS, Stewart FA. Radiation-induced activation of tgf-beta signaling pathways in relation to vascular damage in mouse kidneys. *Radiation Research* 2009;171:188-197.

2. Begg AC, Stewart FA, Vens C. Strategies to improve radiotherapy with targeted drugs. *Nat Cancer Rev* 2011;11:239-253.

3. Hasegawa Y, Takanashi S, Kanehira Y, Tsushima T, Imai T, Okumura K. Transforming growth factor-beta1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. *Cancer* 2001;91:964-971.

4. Huang S, Hölzel M, Knijnenburg T, Schlicker A, Roepman P, McDermott U, Garnett M, Grernrum W, Sun C, Prahallad A, Groenendijk Floris H, Mittempergher L, Nijkamp W, Neefjes J, Salazar R, ten Dijke P, Uramoto H, Tanaka F, Beijersbergen Roderick L, Wessels Lodewyk FA, Bernards R. Med12 controls the response to multiple cancer drugs through regulation of tgf-β receptor signaling. *Cell* 2012;151:937-950.

5. Akhurst RJ, Hata A. Targeting the tgfβ signalling pathway in disease. *Nat Rev Drug Discov* 2012;11:790-811.

6. Zhang M, Herion TW, Timke C, Han N, Hauser K, Weber KJ, Peschke P, Wirkner U, Lahn M, Huber PE. Trimodal glioblastoma treatment consisting of concurrent radiotherapy, temozolomide, and the novel tgf-β receptor i kinase inhibitor ly2109761. *Neoplasia* 2011 13:537-549.

7. Mengxian Z, Kleber S, Roehrich M, Timke C, Han N, Tuettenberg J, Martin-Villalba A, Debus J, Peschke P, Wirkner U, Lahn MM, Huber PE. Blockade of tgfβ signaling by the tgfβr-i kinase inhibitor ly2109761 enhances radiation response and prolongs survival in glioblastoma. *Cancer Res* 2011.

8. XXX

9. XXX

10. XXX

11. XXX

12.XXX.

13. Bristow RG, Hill RP. Hypoxia and metabolism: Hypoxia, DNA repair and genetic instability 2008;8:180-192.

14. Yingling JM, Blanchard KL, Sawyer JS. Development of tgf-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3:1011-1022.

15. Akhurst RJ. Large- and small-molecule inhibitors of transforming growth factor-beta signaling. *Curr Opin Investig Drugs* 2006;7:513-521.

16. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. *Nature* 2009;461:1071-1078.

17. Scagliotti GV, Parikh P, von Pawel J, Biesma B, Vansteenkiste J, Manegold C, Serwatowski P, Gatzemeier U, Digumarti R, Zukin M, Lee JS, Mellemgaard A, Park K, Patil S, Rolski J, Goksel T, de Marinis F, Simms L, Sugarman KP, Gandara D. Phase iii study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol* 2008;26:3543-3551.

18. Shaw AT1, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, Heist RS, Solomon B, Stubbs H, Admane S, McDermott U, Settleman J, Kobayashi S, Mark EJ, Rodig SJ, Chirieac LR, Kwak EL, Lynch TJ, Iafrate AJ. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol 2009; 27: 4247-53.

~~Ettinger DS, Akerley W, Bepler G, Blum MG, Chang A, Cheney RT, Chirieac LR, D'Amico TA, Demmy TL, Ganti AK, Govindan R, Grannis FW, Jr., Jahan T, Jahanzeb M, Johnson DH, Kessinger A, Komaki R, Kong FM, Kris MG, Krug LM, Le QT, Lennes IT, Martins R, O'Malley J, Osarogiagbon RU, Otterson~~

Formatted: Font: 11 pt, Do not check spelling or grammar

Formatted: Font: 11 pt, Do not check spelling or grammar

~~GA, Patel JD, Pisters KM, Reckamp K, Riely GJ, Rohren E, Simon GR, Swanson SJ, Wood DE, Yang SC. Non-small cell lung cancer. *Journal of the National Comprehensive Cancer Network : JNCCN* 2010;8:740-801.~~

19. Villaruz LC, Socinski MA. Personalized therapy for non-small cell lung cancer: Which drug for which patient? *Seminars in Thoracic and Cardiovascular Surgery* 2011;23:281-290.

20. Lo SS, Fakiris AJ, Chang EL, Mayr NA, Wang JZ, Papiez L, Teh BS, McGarry RC, Cardenes HR, Timmerman RD. Stereotactic body radiation therapy: A novel treatment modality. *Nat Rev Clin Oncol* 2010;7:44-54.

21. Asai K, Shioyama Y, Nakamura K, Sasaki T, Ohga S, Nonoshita T, Yoshitake T, Ohnishi K, Terashima K, Matsumoto K, Hirata H, Honda H. Radiation-induced rib fractures after hypofractionated stereotactic body radiation therapy: Risk factors and dose–volume relationship. *International Journal of Radiation Oncology*Biology*Physics*.

22. Diot Q, Kavanagh B, Schefter T, Gaspar L, Stuhr K, Miften M. Regional normal lung tissue density changes in patients treated with stereotactic body radiation therapy for lung tumors. Int J Radiat Oncol Biol Phys. 2012; 84:1024-30.

~~Welcsh P, Lee M, Gonzalez-Hernandez R, Black D, Mahadevappa M, Swisher E, Warrington J, King M. Brca1 transcriptionally regulates genes involved in breast tumorigenesis. Proc Natl Acad Sci USA 2002;99:7560 - 7565.~~

23. D'Angelillo RM, Trodella L, Ciresa M, Cellini F, Fiore M, Greco C, Pompeo E, Mineo TC, Paleari L, Granone P, Ramella S, Cesario A. Multimodality treatment of stage iii non-small cell lung cancer: Analysis of a phase ii trial using preoperative cisplatin and gemcitabine with concurrent radiotherapy. J Thorac Oncol 2009;4:1517-1523.

Formatted: Font: 11 pt, Do not check spelling or grammar

Formatted: Font: 11 pt, Do not check spelling or grammar

Formatted: Line spacing: Double

Formatted: Default Paragraph Font, Font: 11 pt, Do not check spelling or grammar

Formatted: No underline, Font color: Auto, Do not check spelling or

Formatted: Font: 12 pt

Formatted: No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Formatted: Line spacing: Double

Formatted: Font: Calibri, No underline, Font color: Auto

Formatted: Font: Calibri

Linda A, Trovo M, Bradley JD. Radiation injury of the lung after stereotactic body radiation therapy (SBRT) for lung cancer: a timeline and pattern of CT changes. Eur J Radiol. 2011 Jul;79:147-54.

Marquis S, Rajan J, Wynshaw-Boris A, Xu J, Yin G, Abel K, Weber B, Chodosh L. The developmental pattern of brca1 expression implies a role in differentiation of the breast and other tissues. Nat Genet 1995;11:17 - 26.

24. Segawa Y, Kiura K, Takigawa N, Kamei H, Harita S, Hiraki S, Watanabe Y, Sugimoto K, Shibayama T, Yonei T, Ueoka H, Takemoto M, Kanazawa S, Takata I, Nogami N, Hotta K, Hiraki A, Tabata M, Matsuo K, Tanimoto M. Phase iii trial comparing docetaxel and cisplatin combination chemotherapy with mitomycin, vindesine, and cisplatin combination chemotherapy with concurrent thoracic radiotherapy in locally advanced non-small-cell lung cancer: Olcsg 0007. J Clin Oncol 2010;28:3299-3306.Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA, Knuechel R, Kirchner T. Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc Natl Acad Sci U S A 2001;98:10356-10361.

25. Topkan E, Parlak C, Topuk S, Guler OC, Selek U. Outcomes of aggressive concurrent radiochemotherapy in highly selected septuagenarians with stage iiib non-small cell lung carcinoma: Retrospective analysis of 89 patients. *Lung Cancer* 2013;81:226-230..Bhat KP, Balasubramaniyan V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F, Wani K, Heathcock L, James JD, Goodman LD, Conroy S, Long L, Lelic N, Wang S, Gumin J, Raj D, Kodama Y, Raghunathan A, Olar A, Joshi K, Pelloski CE, Heimberger A, Kim SH, Cahill DP, Rao G, Den Dunnen WF, Boddeke HW, Phillips HS, Nakano I, Lang FF, Colman H, Sulman EP, Aldape K. Mesenchymal differentiation mediated by nf-kappab promotes radiation resistance in glioblastoma. *Cancer Cell* 2013;24:331-346.

Formatted: Default Paragraph Font, Font: 11 pt, Not Bold, Do not check spelling or grammar

Formatted: Default Paragraph Font, Font: 11 pt, Not Bold, Do not check spelling or grammar

Formatted: Normal, Justified

Formatted: Font: 11 pt, Not Bold, No underline, Font color: Auto, Do not check spelling or grammar

Formatted: Font: 11 pt, Not Bold, No underline, Font color: Auto, Do not check spelling or grammar

Formatted: Default Paragraph Font, Font: 11 pt, Not Bold, Do not check spelling or grammar

Formatted: Font: 11 pt, Not Bold, No underline, Font color: Auto, Do not check spelling or grammar

Formatted: Font: 11 pt, Do not check spelling or grammar

Formatted: Indent: Left: 0 cm, First line: 0 cm, Space After: 10 pt, Pattern: Clear (White)

Formatted: Font: Not Italic, No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Formatted: Font: Calibri, No underline, Font color: Auto

Formatted: Font: Not Italic, No underline, Font color: Auto

26. Anscher MS, Kong F, Murase T, Jirtle RL. Normal tissue injury after cancer therapy is a local response exacerbated by an endocrine effect of tgfβ. *Brit J Radiat* 1995;68:331-333.

27. Anscher MS, Thrasher B, Zgonjanin L, Rabbani ZN, Corbley MJ, Fu K, Sun L, Lee W-C, Ling LE, Vujaskovic Z. Small molecular inhibitor of transforming growth factor-[beta] protects against development of radiation-induced lung injury. *Int J Radiat Oncol Biol Phys* 2008;71:829-837.

28. XXX

Figure Legends

Figure 1 TGFβ signaling and growth regulation in NSCLC cell lines.

(A) ~~TGFβ production was assessed in 72 hr supernatants from H1299 and H292 cell lines. Total and active TGFβ was determined and normalized to number of cells. (B)~~ Western detection of p-smad2 and total Smad2 in NCI-H1299, NCI-H292, LLC and A549 cells. TGFβ treatment induced phosphorylation of Smad2, which was blocked by LY364947 pre-treatment. Quantitation of the ratios of phosphorylated protein/total protein normalized to TGFβ-treated alone is indicated below each lane. All cell lines were competent to signal via TGFβ receptors. (~~C~~B) Growth of NSCLC cell lines 24 and 48 hr after treatment with small molecular inhibitor LY364947 (SMI), TGFβ, or TGFβ+SMI~~LY364947~~. Values represent ratios of viable cells in untreated control to treatment groups (n=4). Significance was obtained using unpaired, two-tailed Student's t-test; *p<0.05, **p<0.01.

Figure 2 TGFβ inhibition suppresses radiation-induced DNA damage response.

(A) Protein phosphorylation of ATM at serine 1981, Trp53 at serine 15 was assessed by Western for human NCI-H1299, ~~and~~ NCI-H292 and A549 cells and ~~and~~ murine LLC cells. Cells were treated with TGFβ inhibitor LY364947 for 24hr, irradiated with 2 Gy and lysed 30 mins post-IR. (B~~, C~~) TGFβ inhibition with LY364947 significantly decreased γH2AX foci (green) in human NCI-H1299, A549 and NCI-H292 and murine LLC. Nuclei are counterstained with DAPI (blue). Quantification of γH2AX foci shown below each panel reveal a significant reduction in the number of radiationinduced γH2AX foci with LY354947 in NCSLC ($p < 0.0001$; ANOVA). ~~Radiation-induced γH2AX (green) foci were immunostained in monolayers of NCI-H1299 (B) and LLC (C) cells treated with LY364947 for 48hr prior to irradiation with 2 Gy. Nuclear are counterstained with DAPI (blue).~~ Magnification: 40X.

Formatted: Space After: 10 pt, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers

Formatted: No underline, Font color: Auto

Formatted: Font: 12 pt, No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Formatted: Font: 12 pt, No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Figure 3 Unrepaired DNA damage assessed by neutral comet assay.

(A) A representative image is shown for each treatment from the neutral comet assay assessed in LLC cells 4hr after 5 Gy and/or TGFβ inhibitor LY364947 (40x). (B, C) The comet tail moment was measured in LLC cells 30 mins (B) and 4 hr (C) after 5 Gy. Significance was determined using unpaired two-tailed Student's t-test; *$p<0.05$, **$p<0.01$. NS, non-significant

Figure 4 TGFβ inhibitors increase radiosensitivity *in vitro*.

Colony forming efficiency curves are reported for untreated irradiated controls (black squares line) and irradiated cells pretreated with the TGFβ inhibitors (grey circles). Cell lines were treated for 48 hr with LY364947 or TGFβ pan-specific neutralizing monoclonal antibody 1D11 prior to exposure to graded doses of radiation and plated 3 hr post-irradiation for clonogenic

Formatted: Justified

survival analysis. (A) Human NCI-H1299 (B) Human A549 (C) Human NCI-H292 (D) Murine LLC cells treated with LY364947 (E) Murine LLC cells treated 1D11. ~~and (F)~~ The DER_{10} survival is indicated on the graphs. Mean ± S.E. values of triplicate determinations are shown. NCI-H1299, p=0.04; A549, p=0.03; NCI-H292, p=0.33; LLC+1D11,p=0.04; LLC+ LY364947 p=0.03, ANOVA with Tukey post test.

Figure 5 TGFβ inhibition combined with fractionated radiotherapy increases LLC tumor growth delay.

(A) γH2AX (green) foci 1 hr after IR in tumor cells (blue, DAPI) of mice receiving 1D11 TGFβ neutralizing antibody or control antibody 13C4 administered 24 hr prior to tumor irradiation with 2Gy. Fewer foci are evident following RT and 1D11 compared to RT and 13C4. (B) Quantification Mean intensity of γH2AX inside nuclear (~~B~~C) Tumor growth following 5 x 6 Gy daily fractions Averaged tumor growth curves (n=7-8) of in mice treated with 1D11 or 13C4 (mean ± S.D.). (~~C~~D) Tumor growth curves for individual mice are shown. Note delayed regrowth of tumors treated with RT and 1D11. (E) Individual tumor weight at termination. (~~D~~F) Tumor volume change after the first two fractions of 6 Gy RT. *p<0.05 denotes significant differences using ~~analysis of variance (~~unpaired two-tailed Student's t-test~~*ANOVA*.~~).~~

Formatted: Font: 12 pt, No underline, Font color: Auto

Formatted: Font: 12 pt, No underline, Font color: Auto

Formatted: Font: 12 pt, No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Formatted: Font: 12 pt, No underline, Font color: Auto

Formatted: Font: 12 pt, No underline, Font color: Auto

Attenuation of the DNA Damage Response by TGFβ Inhibitors Enhances Radiation Sensitivity of NSCLC Cells *In Vitro* and *In Vivo*

Abstract

Purpose To determine whether TGFβ inhibition increases the response to radiotherapy in human and mouse non-small cell lung carcinoma (NSCLC) cells *in vitro* and *in vivo*.

Methods TGFβ mediated growth response and pathway activation were examined in human NSCLC NCI-H1299, NCI-H292, and A549 cell lines and murine Lewis lung cancer (LLC) cells. Cells were treated *in vitro* with LY364947, a small molecule inhibitor of the TGFβ type I receptor kinase, or with pan-isoform TGFβ neutralizing monoclonal antibody, 1D11, before radiation exposure. DNA damage response was measured by ATM or *Trp53* protein phosphorylation, γH2AX foci formation or comet assay in irradiated cells. Radiation sensitivity was determined by clonogenic assay. Mice bearing LLC syngeneic subcutaneous tumors were treated with 5 fractions of 6 Gy and/or neutralizing or control antibody.

Results NCI-H1299, A549 and LLC NSCLC cell lines pretreated with LY364947 prior to radiation exposure exhibited compromised DNA damage response indicated by decreased ATM and p53 phosphorylation, reduced γH2AX foci, and increased radiosensitivity. NCI-H292 cells were unresponsive. TGFβ signaling inhibition in irradiated LLC cells resulted in unresolved DNA damage. Subcutaneous LLC tumors in mice treated with TGFβ neutralizing antibody exhibited fewer γH2AX foci after irradiation and significantly greater tumor growth delay in combination with fractionated radiation.

Conclusions TGFβ inhibition prior to radiation attenuated DNA damage recognition and increased radiosensitivity in most NSCLC *in vitro* and promoted radiation-induced tumor control *in vivo*. These data support the rationale for concurrent TGFβ inhibition and radiotherapy to provide therapeutic benefit in NSCLC.

Key Words: ionizing radiation; non-small cell lung cancer; TGFβ; small molecule inhibitor; neutralizing antibody

Introduction

Improved treatment of non-small cell lung cancer (NSCLC), which is one of the leading causes of deaths from cancer worldwide, is urgently needed [1]. Approximately 70% of NSCLC patients receive radiotherapy (RT), either alone or in combination with other treatment modalities such as surgery or chemotherapy. Biologically augmenting tumor cell radiosensitivity can play a crucial role in determining treatment success [2]. TGFβ ligands are enriched in the tumor microenvironment, where their production by stromal or tumor cells varies according to the tumor phenotype. In NSCLC, increased TGFβ activity correlates with tumor progression and increased tumor angiogenesis [3,4]. Active TGFβ signaling has been identified as a key factor for chemotherapy resistance in NSCLC [4], in addition to its well-recognized impact promoting tumor progression and metastasis.

TGFβ activation is efficiently induced by ionizing radiation, in part due to the presence of a redox sensitive motif in the latency associated peptide (reviewed in [5]). Growing evidence supports a specific role for TGFβ in mediating the rapid execution of the DNA damage response (DDR) [6]. Normal *Tgfb1* null keratinocyte cell lines irradiated *in vitro* exhibit reduced auto-phosphorylation of ataxia telangiectasia mutated (ATM) protein and its kinase activity [7]. The latter then results in decreased phosphorylation of substrate proteins upon DNA damage, including DNA damage transducers Chk2, p53, and Rad17, and by decreased γH2AX foci, which is a chromatin modification near DNA double strand breaks; together these events impair cell survival. Notably, a TGFβ type I receptor kinase small molecule inhibitor treated human epithelial cells phenocopies murine genetic depletion.

TGFβ signaling blockade augments response to chemoradiation in GBM preclinical models [8,9], and specifically inhibits GBM cancer stem cell renewal after radiation [10]. TGFβ inhibition promotes clonogenic cell death in irradiated mouse and human GBM and breast cancer cell lines in vitro. Systemically neutralizing TGFβ enhances RT actions in GBM and breast cancer preclinical models [8,10,11]. Little is known about the contribution of TGFβ to NSCLC response to radiation. Based on the efficacy in breast and brain tumors, it is plausible that TGFβ inhibition could also improve radiosensitivity of NSCLC.

Currently, TGFβ inhibitors are in clinical trials for several types of cancer including glioblastoma (GBM) and breast cancer (reviewed in [12]). Currently clinically viable inhibitors of TGFβ signaling include small molecule inhibitors of TGFβ type I receptor kinase and neutralizing antibodies that have low toxicity. Clinical trials involving TGFβ inhibition in combination with radiotherapy in patients with breast cancer and GBM are underway (ClinicalTrials.gov identifiers: NCT01401062, NCT01220271). Here, we determined the effect of TGFβ signaling blockade following irradiation in human NSCLC cell lines and murine Lewis lung carcinoma (LLC) tumors. TGFβ inhibition increased radiosensitivity and compromised DDR in 3 of 4 NSCLC cell lines in vitro, and the combination of TGFβ neutralizing antibody with fractionated RT significantly increased LLC tumor growth control in vivo. These results suggest that concurrent TGFβ inhibition and radiotherapy may provide therapeutic benefit in the clinical setting for NSCLC.

Methods

Cell culture

Human NCI-H1299, NCI-H292, A549 and murine LLLC cells were purchased from American Type Culture Collection (Manassas, VA). LLC Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Gibco) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO). Human NCI-H1299, NCI-H292 and A549 were cultured in RPMI-1640 Medium with 10% FBS (Sigma-Aldrich, St. Louis, MO). Cells were treated in 10% serum replacement medium (SRM; Knockout SR, Life Technologies, Inc., Carlsbad, CA) containing either 500 pg/ml TGFβ1 (R&D Systems, Minneapolis, MN), 400 nM small molecule inhibitor of the TGFβ type I receptor kinase, LY364947 ([3-(Pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole; Lilly designation HTS466284; Cat# 616451, Calbiochem, St. Louis, MO) or 10 μg/ml 1D11, a pan-isoform neutralizing TGFβ monoclonal antibody, or 13C4, a murine monoclonal isotype control antibody (kindly provided by Genzyme, Framingham, MA). For growth studies, cells were trypsinized and counted using a Coulter counter at 24-hour and 48-hour post plating.

Clonogenic assay

To assess clonogenic survival of cells in monolayer culture, human and murine lung cancer cell lines were grown for 48 hours to about 70% confluence, at which point media was replaced with serum replacement media. Cells were treated with 400 nmol/L of LY364947 kinase inhibitor or 10 μg/mL pan-specific TGFβ-neutralizing antibody 1D11 or control antibody 13C4 for 48 h before and 3 h post radiation exposure. Cells were irradiated with graded doses of up to 8 Gy using a Varian Clinac 2300 C/D linear accelerator with 1cm bolus below (Varian, Palo Alto, CA). Colony formation of 3 biological replicates was averaged for each treatment and corrected according to plating efficiency of respective controls. Sensitization dose

enhancement ratios (DER10) were calculated as the ratio of doses required to achieve 10% surviving fraction for cells without and with TGFβ inhibition.

γH2AX foci

Tumor cryosections or cells grown on chamber slides were fixed using 2% paraformaldehyde for 20 minutes at room temperature followed by permeabilization with 100% methanol for 20 minutes at −20°C. Then specimens were blocked with the supernatant of 0.5% casein/PBS, stirred for 1 hour, and incubated with a mouse monoclonal antibody against γH2AX (clone JBW301, Upstate Biotechnology, NY) overnight at 4°C as previously described [11]. Specimens were imaged using a 40× objective with 0.95 numerical aperture Zeiss Plan-Apochromat objective on a Zeiss Axiovert (Zeiss) equipped with epifluorescence. All images were acquired with a CCD Hamamatsu Photonics monochrome camera at 1,392 × 1,040 pixel size, 12 bits per pixel depth using the Metamorph imaging platform (Molecular Devices, Inc.).

Comet assay

LLC cells were cultured and treated with 400 nmol/L of LY364947 kinase inhibitor as described above and irradiated with 5 Gy. LLC cells were dissociated 0.5 or 4 hours after irradiation for single-cell gel electrophoresis at 19 V (300mAM, 40 min) and analysis by neutral comet assay (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. SYBR green-stained DNA comets were imaged at 400x magnification and the extent of DNA breaks was quantified as tail moment using Comet Score software.

Western analysis

Cells were grown in complete media for 48 hour, treated with LY364947, irradiated with 5 Gy, and lysed after 1 hr. Protein estimation was carried out using the BCA protein assay kit

from Pierce. One hundred micrograms of protein was electrophoresed on a 4% to 15% gradient gel from BioRad and transblotted on polyvinylidene difluoride membrane. The immunoblots were incubated with one of the following primary antibodies at 1/500 dilution: Smad2 serine 465/467 phosphorylation (Cell Signaling, Danvers, MA), Smad2/3 (BD, Franklin Lakes, NJ), p53 serine 15 phosphorylation (Cell Signaling , Danvers, MA), p53 (Neomarkers, Fremont, CA), ATM serine1981 phosporylation (Epitomics, Burlingame, CA), and ATM, clone 2C1 (GeneTex, Irvine, CA) and detected with infrared labeled antibodies using a LiCor Odyssey system.

Tumor studies

All animal experiments were carried out in accordance with guidelines specified by NYU institutional animal care and use committee. Female 6-8 week old C57BL/6 mice obtained from Taconic were housed in a temperature-controlled animal care facility with a 12-hour light–dark cycle and allowed chow and water *ad libitum*. LLC cells (10^5) were injected into the right flank of mice and allowed to grow until the tumors reached an average size of 60-80 mm^3. Animals were distributed randomly into groups (n=5-10) to receive 1D11 or 13C4 control antibody (10 mg/kg, intraperitoneal injection) 24 hr before localized irradiation and were injected every other day to termination. Radiation was delivered at 600 cGy/min with 6MV X-rays with Varian Clinac 2300 C/D linear accelerator fitted with a 25-mm radiosurgery conical collimator (Varian, Palo Alto, CA). Superflab bolus (1.5-cm tissue equivalent material) was placed over the tumor.

Statistics

Data are presented as mean plus or minus SEM. Statistical comparison between two groups was performed using the Student t test. One-way ANOVA was used to test for

differences among three or more groups. Difference was considered statistically significant, when the p value was less than 0.05.

Results

Response of human and murine NSCLC cell lines to TGFβ

Mutational inactivation of the TGFβ signaling pathway in human NSCLC is associated with specific histological subtypes, more aggressive tumor behavior, and reduced patient survival [2]. NCI-H1299 and NCI-H292 are aggressive tumor cell lines established from a lymph node metastasis of a pulmonary carcinoma, A549 cell line is from a primary human adenocarcinoma and LLC is a highly metastatic murine lung cancer cell line. Since NSCLC cells can selectively evade TGFβ growth regulation while maintaining signaling, we first examined the TGFβ canonical pathway was intact as indicated by Smad2 phosphorylation (p-Smad2) upon exposure to TGFβ. Induction of p-Smad2 by a short TGFβ treatment was suppressed by 50-75% by TGFβ small molecule inhibitor, LY364947 **(Figure 1A)**. These data indicate that receptor-mediated signaling in response to TGFβ is intact in these 4 NSCLC cell lines. Since cell proliferation status confers an important component of the tumor cell radiosensitivity, we tested NSCLC tumor sensitivity to TGFβ-mediated growth inhibition. Only H292 cells were growth-inhibited by TGFβ, whereas NCI-H1299, A549, and LLC cells were refractory to TGFβ-mediated growth regulation **(Figure 1B).**

Blocking TGFβ signaling attenuates DNA damage response in NSCLC cells *in vitro*

We next assessed components of the DDR pathway previously determined to be affected by TGFβ in non-malignant human cells and cancer cell lines [7,10,11]. Phosphorylation of ATM Ser1981 was attenuated after LY364947 SMI treatment in 3 of 4 cell lines. No effect of LY364937 was observed in NCI-H292 cells. Although p53 was undetectable in NCI-H1299 cells

carrying a *Tp53* homozygous deletion, LY364947 significantly decreased phosphorylation of ATM at serine 1981. Both ATM autophosphorylation and p53 phosphorylation at serine 15 was decreased by SMI treatment of LLC cells before irradiation (**Figure 2A)**. As found in our prior studies [10,11], radiation-induced γH2AX foci formation was markedly reduced by pre-treatment with LY364947 in NCI-1299, A549 and LLC cells *in vitro* (**Figure 2B**). Quantitation confirmed the absence of response in NCI-H292 cells (**Figure 2C**).

A compromised DDR could either delay or prevent resolution of DNA double strand breaks [13]. Here we tested the consequences of TGFβ inhibition in LLC cells using neutral comet assays to investigate the effect of TGFβ inhibition on DNA damage induction at 30 min and resolution at 4 hrs following irradiation with 5 Gy (**Figure 3A**). The initial comet tail moment was similar for irradiated cells with or without LY364947, indicating comparable initial DNA damage. Most radiation-induced DSB are repaired in the first 1-6 hr after RT [14]. The comet tail moment decreased compared to 30 min after 5Gy IR. In contrast, the comet tail moment failed to decline in irradiated cells treated with LY364947 (**Figure 3B, C**). This persistent DNA damage indicates that treatment with LY364947 impairs repair of DNA damage.

Inhibition of TGFβ signaling sensitizes NSCLC cell lines to irradiation

The persistence of DNA damage should compromise cell survival. The clonogenic assay is a gold standard for estimating radiosensitivity. Human cell lines NCI-H1299 and A549 were radiosensitized by pre-treatment with LY364947, consistent with the DDR related proteins analysis. Consistent with the lack of SMI effect on DDR, radiosensitization was not evidenced in NCI-H292 cells (**Figure 4 A-C**). Radiosensitization was evident in a large cell lung cancer carcinoma cell line, NCI-H460, when TGFβ was inhibited (data not shown). We next compared

the SMI to a pan-isoform TGFβ neutralizing antibody, 1D11, using the LLC cell line. Murine LLC cells were comparably radiosensitized by pre-treatment with either LY364947 **(Figure 4D**) or 1D11 (**Figure 4E**).

Combination of TGFβ inhibitor with fractionated RT increases LLC tumor growth delay

TGFβ neutralizing antibodies currently in clinical development have demonstrated safety and efficacy in several studies [15,16]. We first determined whether the antibody achieved a biologically effective distribution to affect DDR in tumors by assessing γH2AX foci staining in tumors harvested 1 hr after irradiation with 2 Gy. Similar to the *in vitro* data from LLC cells, induction of γH2AX foci by radiation was reduced in irradiated tumors excised from mice treated with 1D11 compared to control antibody (**Figure 5A and B**). To test the efficacy in combination with RT, we next examined mice bearing LLC subcutaneous tumors treated with TGFβ neutralizing antibodies or in combination with fractionated RT. Subcutaneous LLC tumors were treated with 5 daily fractions of 6 Gy and/or 1D11 or 13C4 (10mg/kg i.p.). Mice treated with 1D11 alone exhibited no significant change in tumor growth compared with control 13C4 antibody treated mice. However, tumor growth delay was significantly enhanced by 1D11 when combined with RT compared to mice receiving RT and 13C4 antibody (**Figure 5C**). Note that tumor regrowth initiated about 5 days following RT was considerably reduced in 1D11 treated irradiated tumors (**Figure 5D**). Tumor volume at the experiment termination (day 27) was significantly smaller for mice treated with 1D11 and RT when compared to RT and 13C4 antibody (**Figure 5E**). Interestingly, more than half (6/11) the mice showed tumor growth rate decrease after the first two fractions of 6Gy when treated with 1D11, in which the mean volume increment was 7.9±6.9 mm^3 (tumor weight 0.74±0.10g). However, tumor growth in

13C4 antibody treated mice had the same growth rate as the non-irradiated tumors, with a mean volume increase of 34.8±9.7 mm^3 (tumor weight 0.34±0.04g) (**Figure 5F)**. These data support the potential benefit of TGFβ inhibition in the context of RT, which attenuates the DDR, radiosensitizes tumor cells, and promotes tumor control *in vivo*.

Discussion

Our study demonstrates that TGFβ inhibition by either pharmaceutical or biological means compromises the DNA damage recognition, repair, and ultimately tumor cell survival in the majority of NSCLC preclinical cell lines. The murine tumor cell line and 2 of 3 the human NCSCL cells were radiosensitized independent of sensitivity to TGFβ-mediated growth inhibition. NCI-H1299 cells, which carry a homozygous deletion of the p53 protein, were still sensitized by TGFβ inhibition, while TGFβ inhibition did not alter DDR or radiosensitivity of NCI-H292, despite the apparent activity of TGFβ signaling. *In vivo* efficacy was demonstrated using LLC syngeneic subcutaneous tumors, where tumor growth control was significantly improved by use of neutralizing antibodies concurrent with fractionated RT. These data suggest that this strategy might be effective in many lung cancer patients.

DNA is the main target for radiation-induced cell killing. Since there is considerable redundancy in the cell's ability to repair DNA damage, modulating the response to ionizing radiation through the inhibition of DNA repair has been a longstanding aim in translational RT research [17]. Previously, we demonstrated that TGFβ is required for efficient ATM activation in epithelial cells [7], that either chemical or genetic inhibition of TGFβ signaling in cells led to reduced ATM activation, and that TGFβ inhibition increased tumor cell radiosensitivity in breast and brain tumor cell lines [10,11]. Consistent with our earlier studies, here we show that TGFβ

inhibition prior to irradiation resulted in reduced phosphorylation of ATM, H2AX and p53 in most cultured NSCLC cells. Moreover, LLC tumors in mice treated with TGFβ inhibitors prior to radiation exposure *in vivo* exhibited less γH2AX foci formation, a nuclear marker of the rapid molecular radiation response and significantly greater growth control following fractionated RT. Interestingly, 1D11 neutralizing antibodies showed immediate tumor growth inhibition after two fractions. At this time point post-IR, tumor cell death results mainly from aberrant repair of radiation-induced DNA damage. This is consistent with a direct effect on radiosensitvity due to compromising the DNA damage recognition. Nevertheless, the exact mechanism or pathways involving this TGFβ inhibition that attenuates IR induced DNA damage responses has not been determined but appears to be p53 independent [11].

NSCLC consists of heterogeneous histologies, with the most common types being adenocarcinoma, large cell carcinoma, and squamous cell carcinoma. The differential response to anti-tumor treatments among these different histologies is a general problem in treating NSCLC [18]. Since most of NSCLC harbor mutations with different clinical characteristics, it is not surprising that till now only one drug, crizotinib used in EML4-ALK–positive patients, has proven to be particularly effective for this specific subpopulation. This “tailored treatment” has resulted in an unprecedented survival benefit [19,20]. Here, we found that TGFβ inhibition had little effect on IR-induced DDR related proteins in NCI-H292, which were not radiosensitized. This suggests that that benefit will be restricted to certain tumors; however, further studies are critical in order to identify the molecular biomarkers that will indicate the potential benefits from inhibiting TGFβ in RT.

Therapeutic resistance and normal lung damage are major challenges for effective RT in NSCLC patients. Hypofractionated stereotactic body radiation therapy (SBRT) allows for escalation of the fractional dose. This is important for not only improving the local control rate of tumors, but the overall survival for medically inoperable patients with early-stage NSCLC [21]. Radiation-induced normal tissue damage is still the biggest obstacle, which limits the dose escalation of SBRT in NSCLC [22-24]. An approach with an enormous appeal involves the development of nontoxic, yet effective, molecularly targeted radiosensitizers that could reduce normal tissue toxicity without affecting control. It is expected that chemotherapy-mediated inhibition of DNA repair mechanisms would synergize the TGFβ inhibition, as shown in combination with Temozolomide and IR in GBM preclinical models [8].If a drug selectively sensitizes the tumor response to radiation, one could use selective escalation of the effective biological dose to the tumor, therefore, improving local tumor control without increasing morbidity. Drugs such as gemcitabine, cisplatin, and docetaxel have been demonstrated to radiosensitize tumors in NSCLC [25]. Although these sensitizers increase the rates of local tumor control and in some cases overall survival [26], they are not specific for tumor cells, and can also increase radiation toxicity to normal tissues. This nonspecific mechanism of action is one of the major limiting factors of many radiation modulators.

Since the lung represents the most RT sensitive tissue, this results in fatal complications. Among the many cytokines involved in this process, TGFβ is thought to play a pivotal role [27,28]. TGFβ is overexpressed at sites of injury, which contributes to delayed tissue fibrosis after radiation and chemotherapy. Various interventional therapies that aim to block TGFβ signaling or decrease tissue levels of TGFβ have proven to be effective in reducing the

development of fibrosis in the lung, liver, and intestines [27-29]. Reduced normal tissue injury and increased tumor control probability by TGFβ inhibition has significant appeal in RT dose-escalation for lung cancer.

Conclusions

Achieving effective targeting the TGFβ pathway is a promising novel treatment option for NSCLC patients, as this selectively increases tumor cell kill and local tumor control, while potentially limiting late effects in the surrounding normal tissue.

Bibliography

1. Kruse JJ, Floot BG, te Poele JA, Russell NS, Stewart FA. Radiation-induced activation of tgf-beta signaling pathways in relation to vascular damage in mouse kidneys. *Radiation Research* 2009;171:188-197.

2. Begg AC, Stewart FA, Vens C. Strategies to improve radiotherapy with targeted drugs. *Nat Cancer Rev* 2011;11:239-253.

3. Hasegawa Y, Takanashi S, Kanehira Y, Tsushima T, Imai T, Okumura K. Transforming growth factor-beta1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. *Cancer* 2001;91:964-971.

4. Huang S, Hölzel M, Knijnenburg T, Schlicker A, Roepman P, McDermott U, Garnett M, Grernrum W, Sun C, Prahallad A, Groenendijk Floris H, Mittempergher L, Nijkamp W, Neefjes J, Salazar R, ten Dijke P, Uramoto H, Tanaka F, Beijersbergen Roderick L, Wessels Lodewyk FA, Bernards R. Med12 controls the response to multiple cancer drugs through regulation of tgf-β receptor signaling. *Cell* 2012;151:937-950.

5. XXX.

6. XXX.

7. XXX.

8. Zhang M, Herion TW, Timke C, Han N, Hauser K, Weber KJ, Peschke P, Wirkner U, Lahn M, Huber PE. Trimodal glioblastoma treatment consisting of concurrent radiotherapy, temozolomide, and the novel tgf-β receptor i kinase inhibitor ly2109761. *Neoplasia* 2011 13:537-549.

9. Mengxian Z, Kleber S, Roehrich M, Timke C, Han N, Tuettenberg J, Martin-Villalba A, Debus J, Peschke P, Wirkner U, Lahn MM, Huber PE. Blockade of tgf-beta signaling by the tgf{beta}r-i kinase inhibitor ly2109761 enhances radiation response and prolongs survival in glioblastoma. *Cancer Res* 2011.

10.XXX.

11.XXX.

12. Akhurst RJ, Hata A. Targeting the tgf[beta] signalling pathway in disease. *Nat Rev Drug Discov* 2012;11:790-811.

13. Thompson LH. Recognition, signaling, and repair of DNA double-strand breaks produced by ionizing radiation in mammalian cells: The molecular choreography. *Mutat Res* 2012;751:158-246.

14. Bristow RG, Hill RP. Hypoxia and metabolism: Hypoxia, DNA repair and genetic instability 2008;8:180-192.

15. Yingling JM, Blanchard KL, Sawyer JS. Development of tgf-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3:1011-1022.

16. Akhurst RJ. Large- and small-molecule inhibitors of transforming growth factor-beta signaling. *Curr Opin Investig Drugs* 2006;7:513-521.

17. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. *Nature* 2009;461:1071-1078.

18. Scagliotti GV, Parikh P, von Pawel J, Biesma B, Vansteenkiste J, Manegold C, Serwatowski P, Gatzemeier U, Digumarti R, Zukin M, Lee JS, Mellemgaard A, Park K, Patil S, Rolski J, Goksel T, de Marinis F, Simms L, Sugarman KP, Gandara D. Phase iii study comparing

cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol* 2008;26:3543-3551.

19. Shaw AT, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, Heist RS, Solomon B, Stubbs H, Admane S, McDermott U, Settleman J, Kobayashi S, Mark EJ, Rodig SJ, Chirieac LR, Kwak EL, Lynch TJ, Iafrate AJ. Clinical features and outcome of patients with non-small-cell lung cancer who harbor eml4-alk. *J Clin Oncol* 2009;27:4247-4253.
20. Villaruz LC, Socinski MA. Personalized therapy for non-small cell lung cancer: Which drug for which patient? *Seminars in thoracic and cardiovascular surgery* 2011;23:281-290.
21. Lo SS, Fakiris AJ, Chang EL, Mayr NA, Wang JZ, Papiez L, Teh BS, McGarry RC, Cardenes HR, Timmerman RD. Stereotactic body radiation therapy: A novel treatment modality. *Nat Rev Clin Oncol* 2010;7:44-54.
22. Asai K, Shioyama Y, Nakamura K, Sasaki T, Ohga S, Nonoshita T, Yoshitake T, Ohnishi K, Terashima K, Matsumoto K, Hirata H, Honda H. Radiation-induced rib fractures after hypofractionated stereotactic body radiation therapy: Risk factors and dose–volume relationship. *International Journal of Radiation Oncology*Biology*Physics*.
23. Welcsh P, Lee M, Gonzalez-Hernandez R, Black D, Mahadevappa M, Swisher E, Warrington J, King M. Brca1 transcriptionally regulates genes involved in breast tumorigenesis. *Proc Natl Acad Sci USA* 2002;99:7560 - 7565.
24. Marquis S, Rajan J, Wynshaw-Boris A, Xu J, Yin G, Abel K, Weber B, Chodosh L. The developmental pattern of brca1 expression implies a role in differentiation of the breast and other tissues. *Nat Genet* 1995;11:17 - 26.

25. Segawa Y, Kiura K, Takigawa N, Kamei H, Harita S, Hiraki S, Watanabe Y, Sugimoto K, Shibayama T, Yonei T, Ueoka H, Takemoto M, Kanazawa S, Takata I, Nogami N, Hotta K, Hiraki A, Tabata M, Matsuo K, Tanimoto M. Phase iii trial comparing docetaxel and cisplatin combination chemotherapy with mitomycin, vindesine, and cisplatin combination chemotherapy with concurrent thoracic radiotherapy in locally advanced non-small-cell lung cancer: Olcsg 0007. *J Clin Oncol* 2010;28:3299-3306.

26. Topkan E, Parlak C, Topuk S, Guler OC, Selek U. Outcomes of aggressive concurrent radiochemotherapy in highly selected septuagenarians with stage iiib non-small cell lung carcinoma: Retrospective analysis of 89 patients. *Lung Cancer* 2013;81:226-230.

27. Anscher MS, Kong F, Murase T, Jirtle RL. Normal tissue injury after cancer therapy is a local response exacerbated by an endocrine effect of tgfb. *Brit J Radiat* 1995;68:331-333.

28. Anscher MS, Thrasher B, Zgonjanin L, Rabbani ZN, Corbley MJ, Fu K, Sun L, Lee W-C, Ling LE, Vujaskovic Z. Small molecular inhibitor of transforming growth factor-[beta] protects against development of radiation-induced lung injury. *Int J Radiat Oncol Biol Phys* 2008;71:829-837.

29. XXX

Figure Legends

Figure 1 TGFβ signaling and growth regulation in NSCLC cell lines.

(A) Western detection of p-smad2 and total Smad2 in NCI-H1299, NCI-H292, LLC and A549 cells. TGFβ treatment induced phosphorylation of Smad2, which was blocked by LY364947 pre-treatment. Quantitation of the ratios of phosphorylated protein/total protein normalized to TGFβ-treated alone is indicated below each lane. All cell lines were competent to signal via TGFβ receptors. (B) Growth of NSCLC cell lines 24 and 48 hr after treatment with small molecular inhibitor LY364947 (SMI), TGFβ, or TGFβ+SMI. Values represent ratios of viable cells in untreated control to treatment groups (n=4). Significance was obtained using unpaired, two-tailed Student's t-test; *$p<0.05$, **$p<0.01$.

Figure 2 TGFβ inhibition impedes radiation-induced DNA damage response.

(A) Protein phosphorylation of ATM at serine 1981 and Trp53 at serine 15 was assessed by Western for human NCI-H1299, NCI-H292 and A549 cells and murine LLC cells. Cells were treated with TGFβ inhibitor LY364947 for 24hr, irradiated with 2 Gy and lysed 30 mins post-IR. Phosphorylation was reduced by at least 25% in 3 of 4 cell lines. NCI-H292 did not show a reduction of phosphorylation. (B) TGFβ inhibition with LY364947 significantly decreased γH2AX foci (green) in human NCI-H1299, A549 and NCI-H292 and murine LLC. Nuclei are counterstained with DAPI (blue). (C) Quantitation of γH2AX foci demonstrates a significant reduction in the number of radiation-induced γH2AX foci with LY354947 in 3 of 4 irradiated NCSLC ($p< 0.0001$; ANOVA). NCI-H292 did not show a reduction of foci. Magnification: 40X.

Figure 3 Unrepaired DNA damage assessed by neutral comet assay.

(A) A representative image is shown for each treatment from the neutral comet assay in LLC cells 4hr after 5 Gy and/or TGFβ inhibitor LY364947 (40x). (B, C) The comet tail moment was measured in LLC cells 30 mins (B) and 4 hr (C) after 5 Gy. Significance was determined using unpaired two-tailed Student's t-test; *p<0.05, **p<0.01. NS, non-significant

Figure 4 TGFβ inhibition increases radiosensitivity *in vitro*.

Radiation survival curves are shown for untreated cells (black squares line) and cells pretreated with a TGFβ inhibitor (grey circles) for 48 hr prior to exposure to graded doses of radiation. Cells were plated 3 hr post-irradiation for clonogenic survival analysis. (A) NCI-H1299 (B) A549 (C) NCI-H292 (D) LLC cells treated with LY364947. (E) LLC cells treated 1D11. The DER_{10} survival is indicated on the graphs. Mean ± S.E. values of triplicate determinations are shown. NCI-H292 was not radiosensitized. Significance was determined using ANOVA with Tukey post test; *p<0.05, NS, not significant.

Figure 5 TGFβ inhibition combined with fractionated radiotherapy increases LLC tumor growth delay.

(A) Immunostaining of γH2AX (green; blue, DAPI) foci 1 hr after 2Gy in tumors of mice receiving 1D11 TGFβ neutralizing antibody or control antibody 13C4 administered 24 hr prior to tumor irradiation. Fewer foci are evident following RT and 1D11 compared to RT and 13C4. (B) Quantification of mean intensity of γH2AX inside mask defined by nuclear DAPI staining. (C) Averaged tumor growth curves (n=7-8) for mice following 5 x 6 Gy daily fractions and/or treated with 1D11 or 13C4 (mean ± S.E.). (D) Individual tumor growth curves for each treatment are shown. Note delayed regrowth of tumors treated with RT and 1D11 compared to RT alone. (E) Individual tumor weights at termination. (F) Tumor volume change after the first two fractions

of 6 Gy RT was evaluated using unpaired two-tailed Student's t-test. Asterisks denote significant differences (*<0.05, **<0.01).

Figure-1

Figure 1

Click here to download Figure: Intl J Rad Onc Bio Phys_R1_Figure-1.pptx

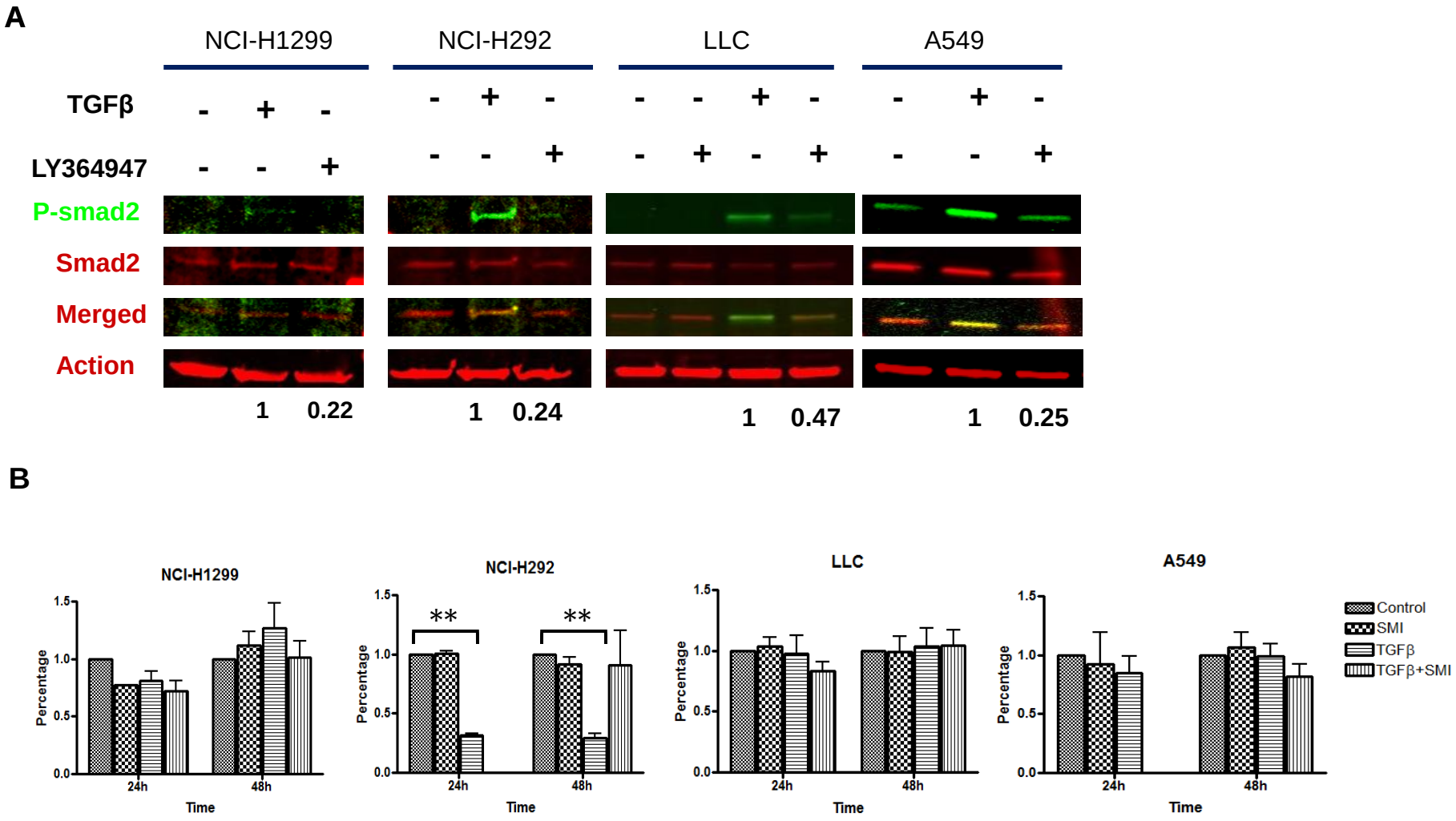

Figure-2A

Figure 2

Click here to download Figure: Intl J Rad Onc Bio Phys_R1_Figure-2.pptx

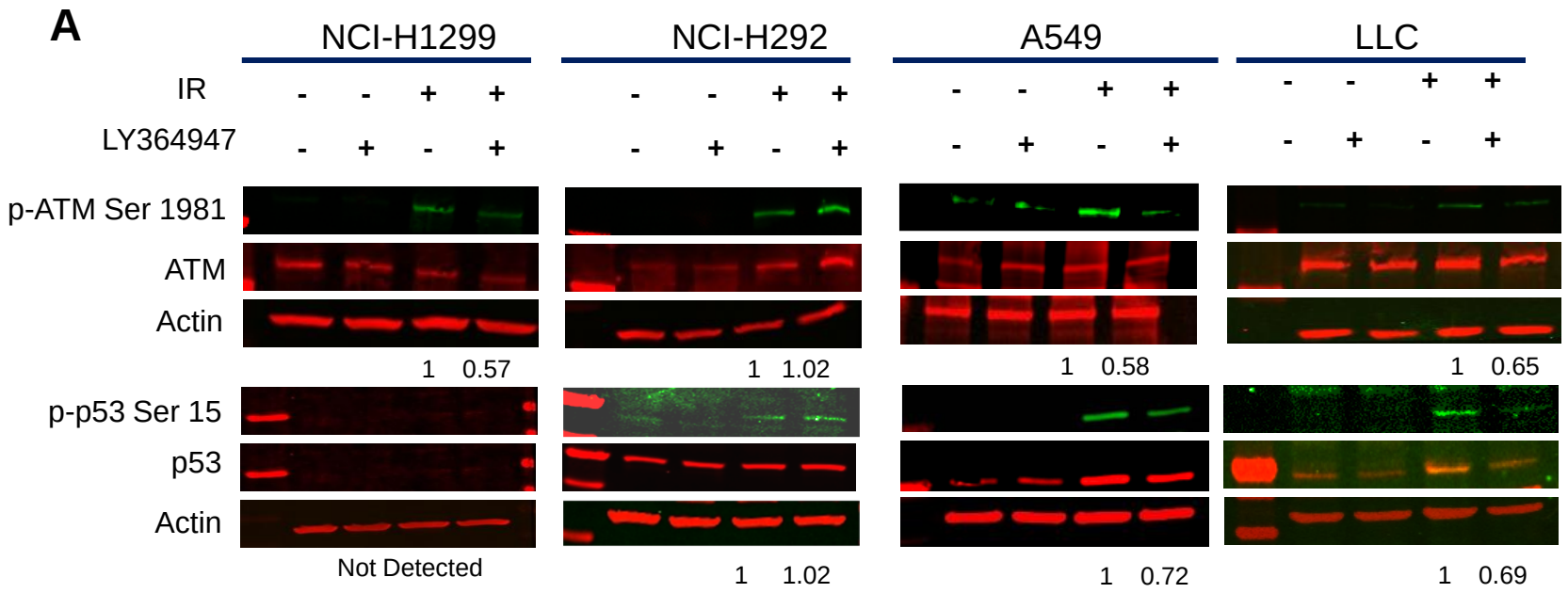

Figure-2BC

Click here to download Figure: Intl J Rad Onc Bio Phys_R1_Figure-2BC.pptx

Figure 2

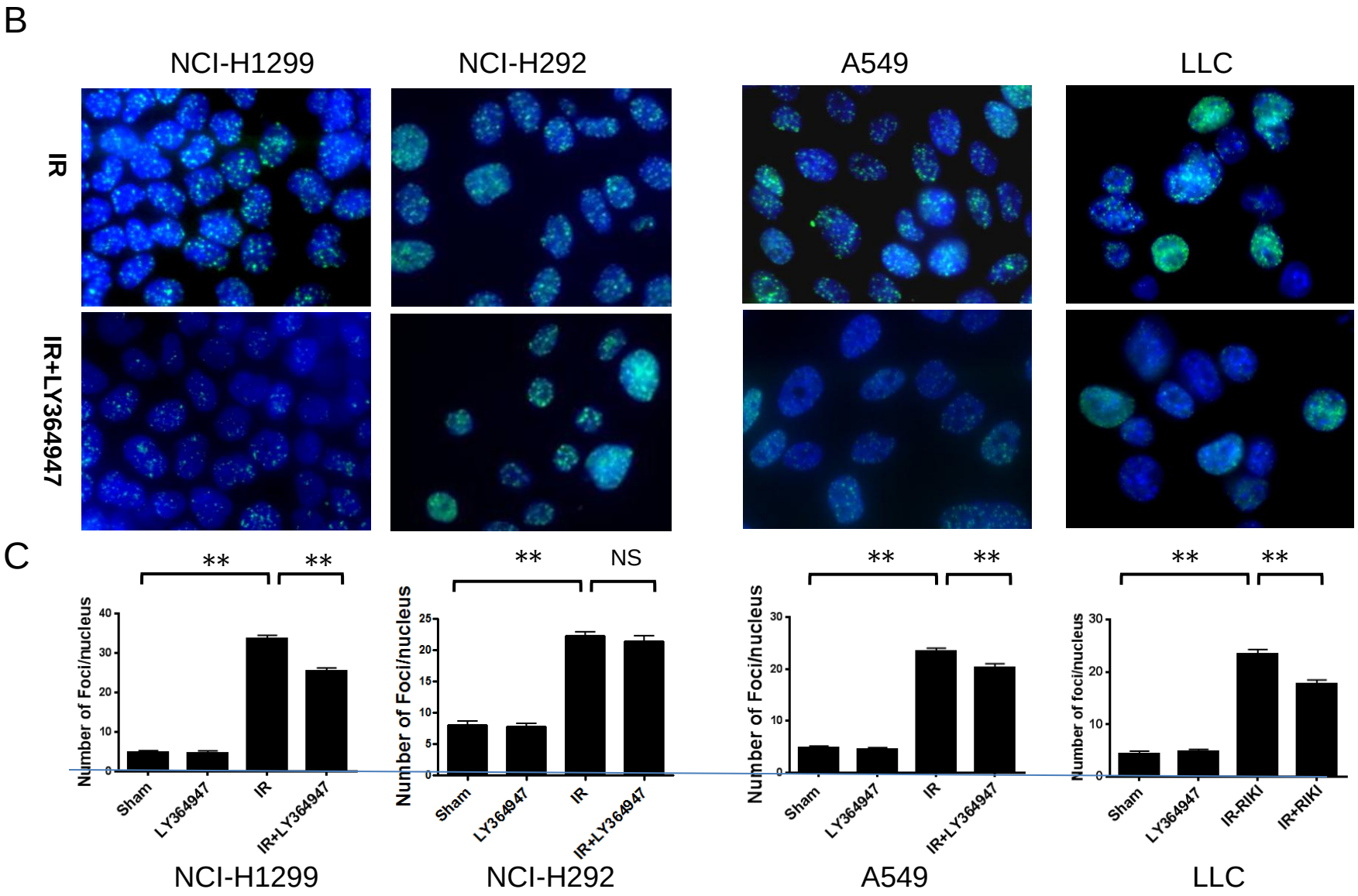

Figure-3

Click here to download Figure: Intl J Rad Onc Bio Phys_R1_Figure-3.pptx

Figure 3

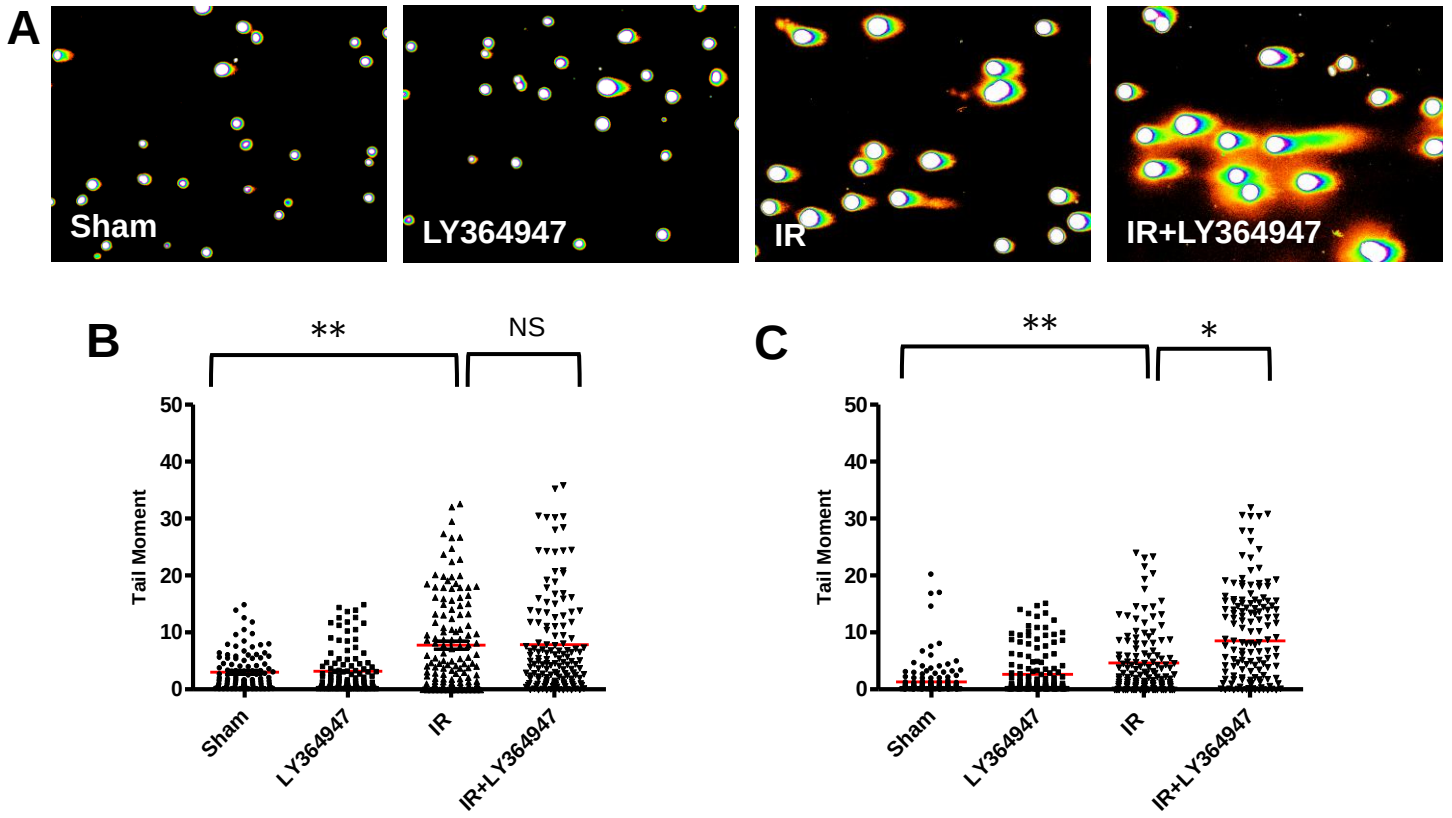

Figure-4

Figure 4

Click here to download Figure: Intl J Rad Onc Bio Phys_R1_Figure-4.pptx

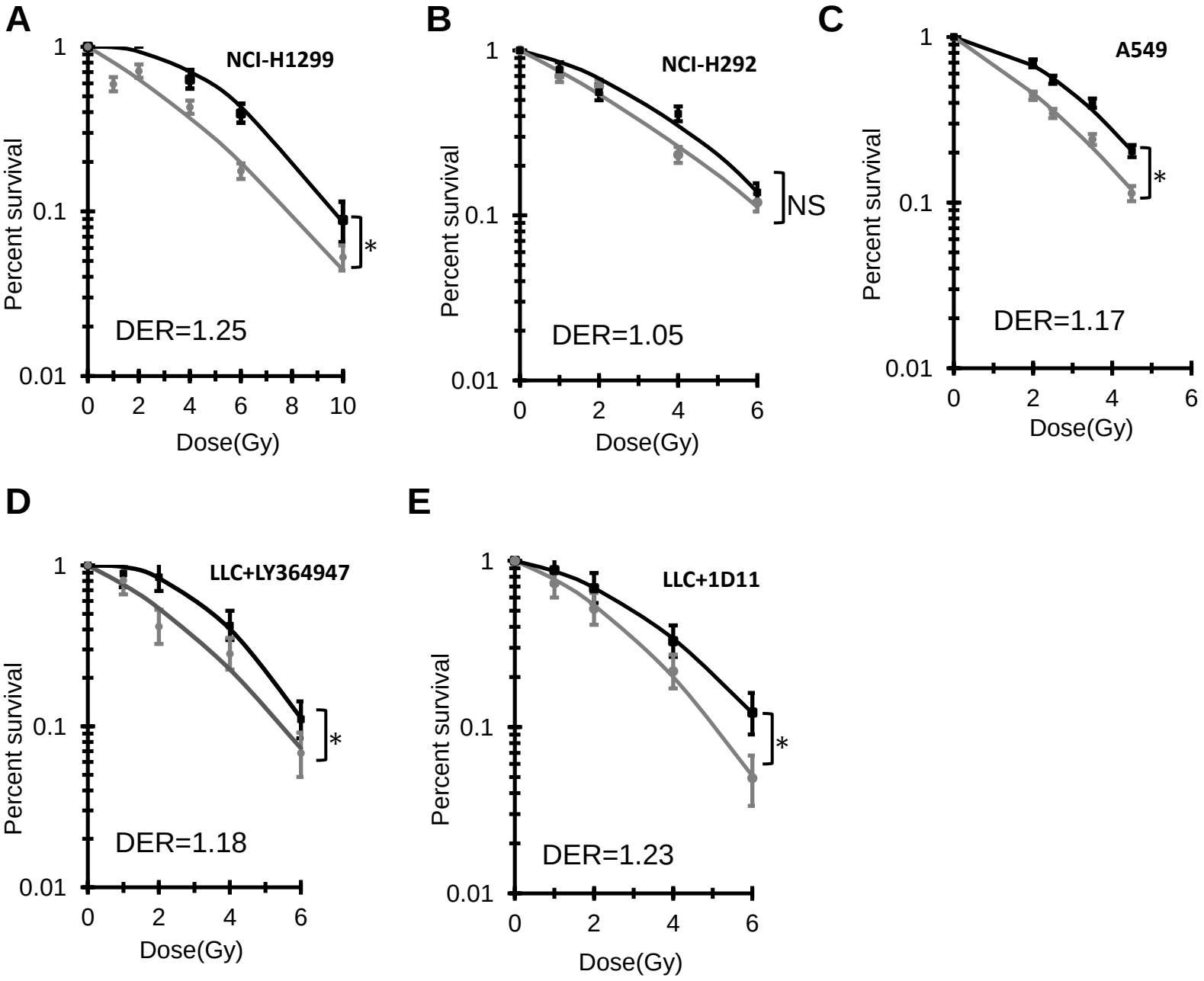

Figure-5

Click here to download Figure: Intl J Rad Onc Bio Phys_R1_Figure-5.pptx

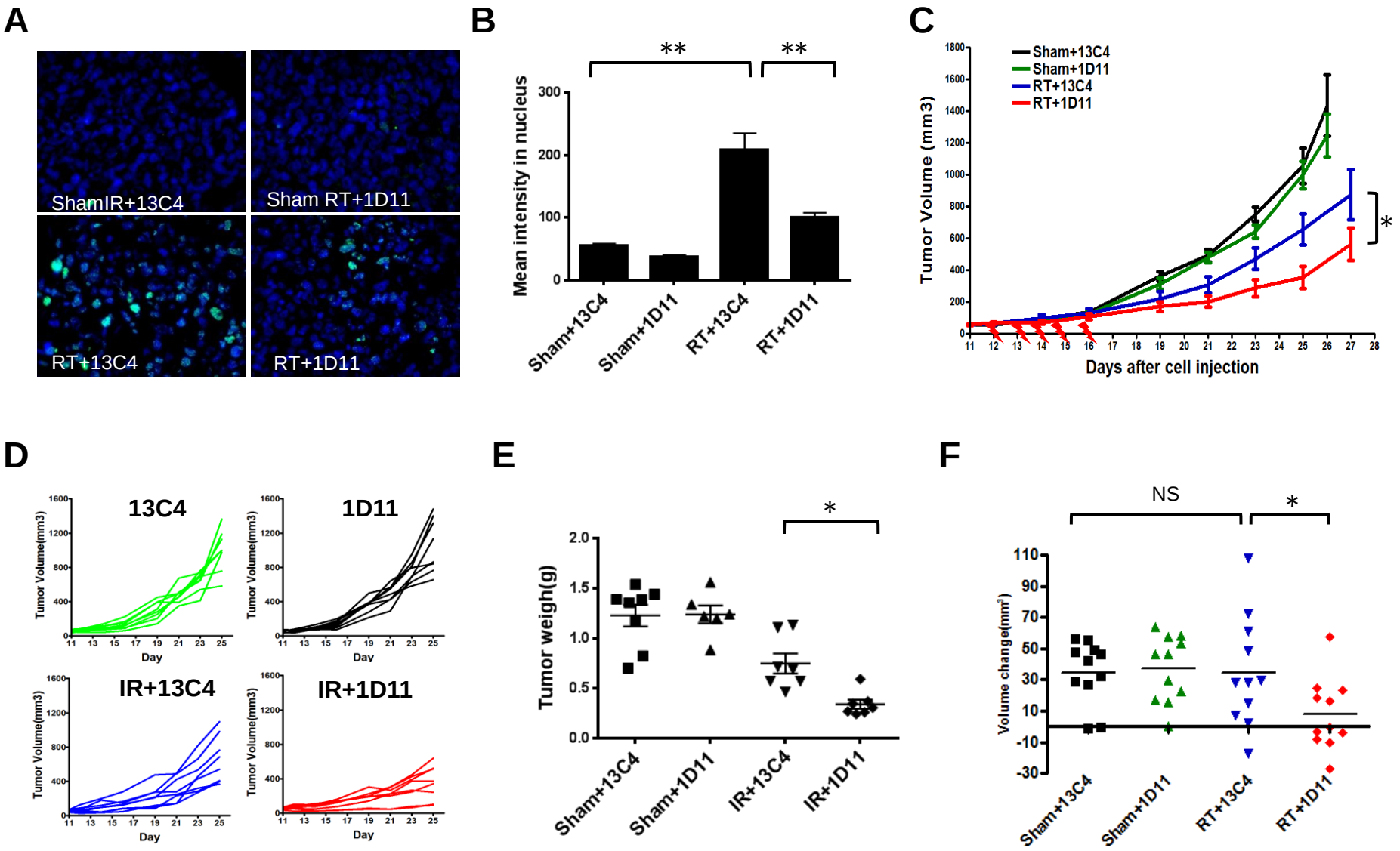

Figure 5

Shisuo Du COI

Click here to download Uniform Disclosures Form: coi_disclosure_Shisuo Du.pdf

Sophie Bouquet COI

Click here to download Uniform Disclosures Form: Sophie Fanny.pdf

Chen-Hao Lo COI

Click here to download Uniform Disclosures Form: coi_disclosure CHEN HAO LO.pdf

Ilenia Pellicciotta COI

Click here to download Uniform Disclosures Form: coi_disclosure_Ilenia.pdf

Shiva Bolourchi COI

Click here to download Uniform Disclosures Form: coi_disclosure_Bolourchi_Shiva.pdf

Renate Parry COI

Click here to download Uniform Disclosures Form: coi_RPdisclosure.pdf

Helen Barcellos-Hoff COI

Click here to download Uniform Disclosures Form: MHBH_disclosure.pdf