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ENHANCING RADIATION SENSITIVITY IN NON-SMALL CELL LUNG CANCER

Yifan Wang

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by

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NON-SMALL CELL LUNG CANCER

A

DISSERTATION

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DOCTOR OF PHILOSOPHY

by

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Houston, Texas

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Dedication

To my beloved wife, Dr. Weiye Deng

Acknowledgments

First and foremost, I would like to express my most sincere appreciation to my mentor, Dr. Steven Lin. He is the person who led me into the field of cancer research. Over the past five years, I have learnt a lot from him, not only about how to do excellent research, but also how to be a good person. Dr. Lin is one of the most hardworking person I have ever seen, his enthusiasm and passion for work inspired me a lot. He is always available whenever I need help or advice, but he also gives me enough freedom and flexibility of research to let me explore this fascinating field. Dr. Lin is a great mentor, I feel very lucky to be his graduate student.

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ENHANCING RADIATION SENSITIVITY IN

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Advisory Professor: Steven H. Lin, M.D., Ph.D.

Lung cancer is the leading cause of cancer-related death. While radiation therapy is one of the standard treatments for lung cancer, the disease outcome after radiotherapy is still far from satisfactory despite ongoing advances in radiation techniques. Enhancing the radiosensitivity of lung cancer has the potential to improve the disease outcome of radiation treatments. Using a novel high throughput radiation sensitizing screen, previous work in the lab has identified several potent radiation sensitizers. The focus of my dissertation is on two of the identified pathways, HSP90 and MEK, using the potent and clinically relevant inhibitors ganetespib and trametinib. Using both *in vitro* experiments in multiple non-small cell lung cancer (NSCLC) cell lines and *in vivo* experiments in animal models, I tested the ability of these inhibitors to radiosensitize lung tumors in the clinically relevant context of chemoradiation. I have found that ganetespib sensitized lung cancer cells to radiation through attenuating DNA damage repair through attenuating DNA damage repair and accentuating G2-M cell cycle arrest. However, when combined with chemoradiation *in vivo*, ganetespib has variable effects on different cells. For radiation sensitization through MEK inhibition, I found that trametinib selectively sensitized KRAS-LKB1 co-mutant NSCLC, but not KRAS-TP53

mutant cells, through radiation-induced senescence. In the LKB1 wild type background, trametinib and radiation activated AMPK-autophagy pathway to rescue cells from senescence, therefore conferring resistance to the radiosensitization. In summary, my studies which focused on how these two specific targeted pathways caused radiation sensitization emphasized the need to better understand the molecular and signaling complexities in determining radiation sensitization effects especially when multiple modalities are combined. Preclinical studies in the context of clinically relevant treatment settings are warranted for optimal clinical translation and personalized cancer therapy.

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Abbreviations

ADP: adenosine diphosphate

ATP: adenosine triphosphate

CAR: chimeric antigen receptor

CRISPR: clustered regularly interspaced short palindromic repeats

CRT: chemoradiation

CTLA-4: cytotoxic T-lymphocyte–associated antigen 4

EGFR: epidermal growth factor receptor

GAP: GTPase-activating protein

HMGB1: high mobility group box 1

HSP: heat shock protein

IFN: interferon

IHC: immunohistochemistry

IMRT: intensity-modulated radiation therapy

LKB1: liver kinase B1, also known as serine/threonine kinase 11 (STK11)

MDM2: mouse double minute 2

MHC: major histocompatibility complex

NSCLC: non-small cell lung cancer

OVA: ovalbumin

PARP: poly (ADP-ribose) polymerase

PCR: polymerase chain reaction

PD-1: programmed death-1

PD-L1: programmed death-ligand 1

SCLC: small cell lung cancer

STING: stimulator of interferon genes

TGF: transforming growth factor

TKI: tyrosine kinase inhibitor

VEGF: vascular endothelial growth factor

Chapter 1: Introduction

1.1 Lung cancer epidemiology

Lung cancer is the leading cause of cancer-related death in both men and women (4). In the United States, lung cancer is estimated to cause more than 150,000 deaths in 2017, more than the total deaths from prostate, breast, and colon cancers combined (4). The mean age of lung cancer patients when diagnosed is over 70, and more than 65% of the patients are older than 65 (5). The survival for lung cancer decreases with age for both sexes (5). The risks associated with lung cancer include smoking, air pollution, occupational exposure, and genetic mutations (6). The incidence of lung cancer deaths has dropped in the 1990s mainly due to the reduction in tobacco use. However, lung cancer deaths are estimated to continue to rise worldwide, especially in developing countries due to larger prevalence of tobacco use and air pollution (4, 5).

Lung cancer can be divided into two categories, non-small-cell lung cancer (NSCLC), and small cell lung cancer (SCLC) (5).

SCLC accounts for 15% of lung cancer. SCLC is clinically and biologically different from other lung cancers, with the majority of cells expressing neuroendocrine signatures (7). SCLC is usually diagnosed at late stage. Although SCLC is sensitive to cytotoxic therapies, recurrence is very common and always with resistance to further therapies (7).

About 85% of lung cancer is non-small-cell lung cancer (NSCLC) which can be further divided into three pathologic subtypes: adenocarcinoma (38.5%), squamous cell carcinoma (20%), and large cell carcinoma (2.9%) (8). Smoking can induce all types of lung cancer, but it is most commonly associated with squamous cell carcinoma and

small cell lung cancer. For never smokers, adenocarcinoma is the most common lung cancer type (8).

The 5-year survival of lung cancer in China, Europe, and developing countries is estimated at 9% (5). In the United States, the 5-year survival of lung cancer is about 15%, most of which are due to the fact that more than half of the lung cancers were diagnosed with distant disease, and the 5-year survival rate for these patients is less than 5% (4).

1.2 Molecular characteristics of non-small cell lung cancer

A number of tumor-specific mutations are associated with lung cancer. These mutations confer distinct characteristics and response to therapies. Molecular profiling of tumors could help guide treatment which may improve the outcomes of NSCLC patients (8). It is therefore imperative to better understand the molecular mechanisms underlying the aberrant genes in NSCLC. Common genetic mutations detected in lung cancer includes *TP53*, *KRAS*, *EGFR*, and *LKB1* (9).

I*53* is a tumor suppressor gene that plays important roles in genome stability, DNA damage response, senescence, apoptosis, and cell cycle regulation (10). The p53 signaling pathway is activated by several kinds of stress signals. The protein level of p53 is strictly regulated by MDM2, an E3 ubiquitin ligase. In cells without stress, MDM2 monoubiquitinates p53. Once cells are under stress, the interaction between p53 and MDM2 will be disrupted, stabilizing the p53 protein to trigger downstream signals (11). More than 75% of p53 mutations are loss of function mutations, with some p53 mutations are gain of function mutations which render oncogenic functions of p53, such

as the Li-Fraumeni mutation (12). Directly restoring wildtype p53 expression through transfection or retrovirus infection was previously shown to have anti-cancer effects (13, 14). The *p53* gene therapy drugs, Gendicine and Ocorine, were approved in China in 2003 and 2005 (15).

KRAS is the most frequently mutated oncogene in NSCLC outside of Asia (9). When KRAS binds to ATP, it is in activated form to phosphorylate and activate downstream signaling pathways. Once the ATP is dephosphorylated to ADP by GAP, KRAS signaling is shut down. Mutation of KRAS is most common at G12 or G13, which is near the ATP binding pocket. These mutations blocks the interaction between GTP and GAPs, therefore KRAS is "locked" at the activated form to sustain activate downstream pathways to promote cell growth, proliferation and render resistant to apoptosis (16). Efforts to inhibit KRAS directly by molecular targeting drugs have failed for decades until recently (17, 18), however the clinical efficacy still needs to be fully evaluated. Alternatively, drugs target downstream of KRAS signaling are effective in some clinical trials (19).

EGFR is the most common mutation in lung cancer patients in Asia, consisting 30% to 50% among all cases. In the United States, EGFR mutation rate is approximately 10% (20). EGFR is an important regulator of cell proliferation, invasion, angiogenesis, and apoptosis (21). EGFR mutations are associated with increased sensitivity and responsiveness to EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib (22, 23). However, relapse is common among the patients who initially responded to erlotinib and gefitinib. Mechanisms that induce resistance include secondary mutation of EGFR, mutation of downstream KRAS, activation of insulin-like growth factor 1 receptor, and epithelial-mesenchymal transition (16, 24-26).

LKB1 is a sensor of cellular energy status and also functions as a tumor suppressor which is mutated in 15%-30% of NSCLC (27). Germline mutation of LKB1 could lead to Peutz–Jeghers syndrome (28). When the ATP/ADP ratio in cell is low, LKB1 is activated and inhibits cell growth through the downstream AMPK pathway. Because LKB1 directly regulates the AMPK pathway to control metabolism and growth of the cell. LKB1-mutant cells are resistant to metabolic stress (29). The mutation of *LKB1* in lung cancer patients is strongly associated with *KRAS* mutation and smoking history (30). Although existing studies of *LKB1* have not found a significant association between its mutation with overall survival of NSCLC patients (30, 31), several studies have illustrated the relationship between *LKB1* and response to therapies. The study of Richer et al. showed *Kras-Lkb1* deficient lung cancer cell lines and genetically modified mouse models were sensitive to WEE1 kinase inhibitor and the combination of WEE1 inhibitor with DNA-damaging agents, such as cisplatin. Their results also demonstrated that restoring the expression of wild type *Lkb1* reduced sensitivity to WEE1 inhibitor (32). Another study published by Liu et al. identified synergistic inhibition of *LKB1*-decifent lung cancer by the combination of gemcitabine and Chk1 inhibitor AZD7762 (33). Importantly, recent studies suggest *LKB1* is a critical modulator of cancer immunotherapy response. In a preclinical study of *Kras*-mutant lung cancer mouse model, *Lkb1* knockout increased neutrophil accumulation in the tumors and enhanced expression of T-cell exhaustion markers. Additionally, compared with *Kras* mutant tumors, *Kras-Lkb1* mutant tumors expressed lower levels of PD-L1 and had decreased number of tumor-infiltrating lymphocytes. As a result, Kras-Lkb1 tumors were not responsive to PD-1 blocking antibodies (34). Another preclinical study showed synergistic effects of radiation and anti-PD-1 in *Kras*-driven mouse model of lung cancer, but the synergy was abolished by additional ablation of *Lkb1* (35).

Studies in the recent decade indicated the KRAS-mutant NSCLC is a genetically and functionally complex disease. Distinct subtypes exist among this type of lung cancer. Actually, in the KRAS-mutant NSCLC there are often other genes co-mutated with KRAS, the most frequent ones among them includes p53 and LKB1 (9). Bioinformatics and functional studies reveals that the p53 and LKB1 co-mutations in KRAS-mutant non-small cell lung cancer defines the major subtypes of this genetically complex disease (36). These KRAS-mutant NSCLC subtypes have differential gene expression profiles and responses to therapies, including chemotherapies, radiation, targeted therapies, and immunotherapies (36-39). Chen et al. showed that the effect of docetaxel could be enhanced by the MEK inhibitor, selumetinib, in *Kras* and *Kras-p53* mouse lung cancer models, however not in *Kras-Lkb1* model (38). Herter-Sprie et al. induced single lung tumor modules in genetically modified mouse models with *Kras*, *Kras-p53* or *Kras-Lkb1* mutations by injecting Adeno-Cre and treated the tumors by radiation. Their data showed radiation effectively controlled tumor growth in *Kras* and *Kras-p53* tumor models, however, the *Kras-Lkb1* tumors grew rapidly after 4 weeks of treatment (37). The study by Shackelford et al. in 2013 showed the metformin analog, phenformin, selectively induced apoptosis of *Kras-Lkb1* mutant NSCLC cell lines and prolonged survival of *Kras-Lkb1* lung cancer mouse model, however such therapeutic effects of phenformin was not observed in NSCLC bearing *Kras-p53* mutations (40). In addition to preclinical studies, the differences between *KRAS-p53* and *KRAS-LKB1* NSCLC were also found in human cancers. In a genomic study of *KRAS*-mutant NSCLC in The Cancer Genome Atlas (TCGA), *KRAS-p53* and *KRAS-LKB1* were identified as two subgroups with distinct gene expression profile and responses to therapies (36). Therefore, specific therapeutic strategies should be developed for each subtype.

1.3 Radiation therapy in cancer treatments

Radiation is one of the standard treatments for lung cancer (6). In the past few decades, advances in radiation technology and improved understanding of radiation biology has enabled radiation oncologists to deliver radiation more precisely and provide personalized treatments (41). For example, the state-of-art technologies such as intensity-modulated radiation therapy (IMRT) and proton radiation could precisely deliver radiation to the site of tumor while minimizing the dose exposure to surrounding normal tissues (41). The tumor radiosensitivity are mediated by the biological mechanisms including hypoxia and reoxygenation, repopulation between fractions, redistribution of cell cycle population, and repair of DNA damage (41-45). Today, many patients treated with radiotherapy received combined modalities, such as chemotherapy, targeted therapy, and immunotherapy, to achieve better treatment efficacy (46). For example, radiotherapy is usually combined concomitantly with chemotherapy for unresectable NSCLC with curative intent. Chemoradiotherapy (CRT) produces longer overall survival than sequential chemotherapy and radiation therapy (47-51). However, the toxicity remains high and the outcomes are still not satisfying. We need a more in-depth understanding of the mechanisms underlying radiosensitivity of tumor to improve the current treatment standards.

1.4 Radiation sensitization

Despite advances in radiation delivery, the efficacy of radiation for treating lung cancer still has much room for improvement, particularly for locally advanced cancers receiving

chemoradiation therapy since local recurrence after chemoradiation is in the order of 30-40%. Thus, sensitizing tumor cells with molecularly targeted agents holds the promise of achieving higher probability of tumor control by using the same or even lower radiation doses. Many drugs, including chemotherapy, monoclonal antibodies, and targeted agents, have radiosensitizing effects in several tumor types (52-57). Several mechanisms could be responsible for radio-sensitization. First, the molecular target may be differentially expressed in tumor vs normal tissues, so targeting a tumor-specifically expressed target may generate differential toxicity in tumors. Second, some drugs, for example DNA repair machinery inhibitors, are not highly toxic to tissue but can be synthetically lethal with radiation. The inhibition of DNA repair machinery during or after the course of radiation causes significant stress to inhibit cancer cell growth or induce apoptosis. For example, PARP inhibitors have been shown to be radiosensitizing (58). Third, although drugs act systemically, radiation is delivered precisely to specific regions of the tumor, leading to more targeted killing of tumor cells while minimizing damage to normal tissue.

The overall goal of my research projects is to enhance the radiosensitivity of NSCLC to enhance the treatment outcome of radiotherapy. Due to the genetic complexity of the different NSCLC subtypes, identifying biomarkers to predict response to the radiosensitizing therapies is an important need for personalized radiotherapy.

Chapter 2: Radiosensitize NSCLC by HSP90 Inhibitor Ganetespib

2.1 Introduction

Adapted from previously published work by Wang et al., ***Hsp90 Inhibitor Ganetespib Sensitizes Non–Small Cell Lung Cancer to Radiation but Has Variable Effects with Chemoradiation,*** *Clinical Cancer Research, 2016, doi: 10.1158/1078-0432.CCR-15-2190.*

Lung cancer is the leading cause of cancer death in the United States and has a 5-year relative survival rate of only 16% (59). Non-small cell lung cancer (NSCLC) accounts for about 85% of all lung cancer cases and the lack of significant treatment advance is related to the highly resistant nature of this disease. While chemotherapy provides only useful palliation for stage IV NSCLC, the treatment of locally advanced, unresectable NSCLC is with curative intent using concomitant chemotherapy and radiotherapy (chemoradiotherapy, CRT), which produces longer overall survival than sequential chemotherapy and radiation therapy (47-51), but the outcomes remain poor. The median survival ranges from 17-28 months, despite significantly increased toxicity of the combination therapy. There is a strong need to improve therapy efficacy in NSCLC without substantially increasing normal tissue toxicity. Indeed, recent clinical trials have investigated the combination of CRT with molecularly targeted agents, either with angiogenesis inhibitors or with EGFR targeting agents. Unfortunately, either due to

intolerable toxicities (60) or to lack of efficacy (61), these trials have not advanced the management of this disease.

Hsp90 is a molecular chaperone protein ubiquitously present in cells; however its function is critically important for the maintenance of cancer cell growth (62, 63). Inhibiting its function has been extensively studied for its potent antitumor effect (62, 64, 65). An attractive feature of targeting Hsp90 is that the cytotoxicity of Hsp90 inhibitors is tumor selective (66). Hsp90 inhibition has also been known to be radiation sensitizing on tumor cells (67-78). Some of these studies indicated that the radiation sensitization is also tumor selective since normal cells are not affected (67, 68, 70). However, the clinical development of Hsp90 inhibitors has been hampered by the severe toxicities of first generation inhibitors, including severe ocular and hepatic toxicities (79, 80).

Ganetespib, a second generation Hsp90 inhibitor with little to no ocular or hepatic toxicities, has been safely used in thousands of patients in over 60 clinical trials internationally. A completed phase II randomized trial in stage IV NSCLC combining ganetespib with docetaxel compared to docetaxel alone has demonstrated efficacy signal in a subgroup of patients (81), and therefore it was further tested in a phase III randomized trial (GALAXY II) (NCT01798485). Despite the safety and promising efficacy of this drug in advanced NSCLC, the experience of combining ganetespib with CRT is limited. While radiation sensitizing effect is well known for this class of inhibitors, one simply cannot assume that synergy could be seen with CRT. This was once assumed for EGFR inhibitors when preclinical studies demonstrated synergy with radiotherapy alone (82, 83), but ultimately failed in a number of phase III clinical trials when EGFR targeting agents were combined with CRT in oropharyngeal cancer, esophageal cancer, and NSCLC (61, 84, 85). While this manuscript was under preparation, Gomez-Casal et al.

reported the HSP90 inhibitor ganetespib radiosensitizes human lung adenocarcinoma cells (86), however whether ganetespib enhanced therapeutic effects of chemoradiotherapy was not demonstrated. The purpose of the current study is to evaluate the cytotoxic action of the combination of radiation with ganetespib and test its potential to synergize with CRT for the treatment of NSCLC.

2.2 Materials and Methods

Adapted from previously published work by Wang et al., ***Hsp90 Inhibitor Ganetespib Sensitizes Non–Small Cell Lung Cancer to Radiation but Has Variable Effects with Chemoradiation,*** *Clinical Cancer Research, 2016, doi: 10.1158/1078-0432.CCR-15-2190.*

2.2.1 Cell culture, reagents and irradiator

The human non-small lung cancer cell lines H460, A549, H1299, and H1650 cells were all obtained from the American Type Culture Collection (ATCC) and routinely maintained in RPMI-1640 medium supplemented with 10% FBS, and 10,000 U/mL of penicillin-streptomycin. Ganetespib [3-(2,4-dihydroxy-5-isopropylphenyl)-4-(1-methyl-1H-1,2,4-triazol-5(4H)-1] was provided by Synta Pharmaceuticals Corp. Cells and animals were irradiated with a JL Shepherd Mark I-68A 137Cs irradiator with 137Cs sources at the doses from 0-6 Gy. The Cesium-Irradiator output (cGy/min) was measured in-air using an ADCL (Accredited-Dosimetry-Calibration-Laboratory) calibrated ion-chamber. Dosimetry in Simulated-Irradiation-Geometry was performed employing Gafchromic-Film “EBT3”. For Film-Dosimetry, mouse was simulated by dosimetrically-equivalent Gel “SuperFlab”. EBT3 response in Simulated-Geometry versus in-Air Reference-Calibration-Geometry provided dose-rate in mouse. The treatment set-up employed table, cerrobend-blockand mouse-

restrainer on top. Cesium beam pointed up. All these pieces were provided with mutually inter-locking pins to ensure set-up reproducibility. For reduced penumbra, the block was provided with appropriate divergence.

2.2.2 Clonogenic survival assay to determine ganetespib radio sensitization effect

The effectiveness of the combination of ganetespib and ionizing radiation was assessed by CSA. H460, A549, H1299, and H1650 cells were seeded (100-2000 cells/well) in duplicate in 6-well plates. The medium was changed 16 hours after plating and the cells were treated with either vehicle (DMSO) or ganetespib (30 nM). Five hours following ganetespib treatment, the cells were subjected to irradiation at doses from 0-6 Gy. Twenty-four hours after ganetespib treatment, media was changed and the cells were maintained in normal culture conditions. On about 12th-20th day, the medium was removed and cell colonies were stained with crystal violet (0.1% in 20% methanol) (Sigma-Aldrich, St. Louis, MO, USA). Colony numbers were assessed visually and colonies containing > 50 normal-appearing cells were counted. The surviving fraction was calculated using SigmaPlot 10.0 (San Jose, CA, USA).

2.2.3 Clonogenic survival assay for chemoradiation with ganetespib

The combination effect of chemo radiation and ganetespib was assayed in vitro using clonogenic survival assay. Cells were seeded in 6-well plates 16 hours prior to treatment. The cells were treated by chemo (Paclitaxel 3.51 nM with

Carboplatin 24.23 nM) and/or ganetespib (30 nM) followed by radiation (2 Gy) after 4-5 hours. Drugs were washed out 24 hours after the treatment and the cells were maintained in normal cultural conditions for 12-15 days. The colonies were stained by crystal violet (0.1% in 20% methanol) (Sigma-Aldrich, St. Louis, MO, USA). Colony numbers were assessed visually and colonies containing > 50 normal-appearing cells were counted. The surviving fraction was calculated using GraphPad Prism 6 (La Jolla, CA, USA).

2.2.4 DNA repair foci formation assay

H460 and A549 cells were grown as monolayers on chamber slides with plastic bottom (Nunc Lab-Tek, Roskilde, Denmark) and were treated with DMSO or ganetespib (25 nM, 50 nM) 24 hours after seeding into culture chambers. Five hours after ganetespib treatment, the cells were subjected to irradiation at dose of 2 Gy. Thirty minutes, 4 hours, 24 hours and 48 hours after irradiation, cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and washed in PBS. The cells were then permeabilized in 0.5% Triton X-100 for 10 min, and blocked in PBS with 3% BSA (Bovine serum albumin) for 20 min at room temperature. The cells were sequentially incubated with anti-53BP1 antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:100 dilution overnight at 4℃ and Alexa Fluor® 488 Conjugate secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at a 1:1000 dilution for 1 hour at 37°C in PBS with 1.5% BSA and washed three times in PBS. Nuclei were counterstained with 1:500 4-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS. Cover glasses were mounted in

Vectashield (Vector Laboratories). Fluorescence images were captured with Leica fluorescence microscope equipped with a CCD camera and images were imported into Advanced Spot Image analysis software. DNA repair foci were quantified using ImageJ software (NIH public domain).

2.2.5 Flow cytometry cell cycle analysis

H460 and A549 cells were treated with vehicle (DMSO) or ganetespib (50 nM) with or without irradiation as described above and were harvested 4 hours, 16 hours, 24 hours, 48 hours, 72 hours and 96 hours after irradiation and fixed in 70% ethanol for 1 hour at 4°C. The cells were stained with propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) for 15 min in the dark and FACS analysis was performed using a Becton-Dickinson FACS Calibur flow cytometer (BD Biosciences, Heidelberg, Germany) per the manufacturer's instructions. Assays were performed in triplicates and the representative results were shown in the figures. The percentage of cells in each phase of the cell cycle (sub-G1, G1, S and G2/M) was determined with Flow-Jo (TreeStar Inc., Ashland, OR).

2.2.6 *In vivo* NSCLC xenograft model

Female NCr nu/nu mice (5 weeks old, from Taconic Biosciences, Germantown, NY, USA) were used for tumor studies. Animals were maintained in an Association for the Assessment and Accreditation of Laboratory Animal Care approved facility in accordance with current regulations of the U.S. Department

of Agriculture and Department of Health and Human Services. Experimental methods were approved by and in accordance with institutional guidelines established by the Institutional Animal Care and Use Committee. For H460 xenografts, mice were subcutaneously inoculated with a total of 1 X 106 H460 cells in 20 ul PBS into their right hind legs to prepare the tumor model. For A549 xenografts, mice were subcutaneously inoculated with a total of 5 X 106 A549 cells in 50 ul PBS to the right hind legs. When the tumor reached 100 mm3 (H460) or 150 mm3 (A549), mice were randomized into different treatment groups (4-10 animals per group). The treatment schedule of both models is described as below. Mice were treated with Carboplatin (30mg/kg)-Paclitaxel (10mg/kg) intraperitoneally one time weekly for two weeks and/or intravenously via the tail vein with 100 mg/kg ganetespib formulated in about 200 ul 10/18 DRD (10% DMSO, 18% Cremophore RH 40, 3.8% dextrose) once weekly for two weeks, other mice not in the Ganetespib treatment groups received the same volume of solvent. Five hours after drug treatment, irradiation was applied locally to the tumor-bearing legs of unanesthetized mice at 2 Gy once daily for 5 days. Tumor volumes (V) were calculated by caliper measurements of the width (W), and length (L) of each tumor using the formula: V=0.5 X (L X W2). The tumor growth curve was calculated using GraphPad Prism 6 (La Jolla, CA, USA).

2.2.7 Immunohistochemistry

Tumors were fixed in 1:10 diluted formalin (Fisher scientific, Cat# 245-684, Waltham, MA USA) overnight and embedded in paraffin. Tissue sections (2

mm) were deparaffinized in 100% xylene and rehydrated through incubation in descending ethanol dilutions (100–60%) followed by boiling at 125℃ for 2 min in Citrate buffers (10 mM Sodium Citrate pH 6). To reduce the endogenous peroxidase activity, slides were treated with 3% H2O2 for 10 min and subsequently probed with the primary antibody anti-cleaved caspase 7 (Cell Signaling Technology, Danvers, MA, USA) at 1:1000 dilution and secondary antibody SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling Technology, Danvers, MA, USA). DAB (SignalStain® DAB Substrate Kit, Cell Signaling Technology, Danvers, MA, USA) was used as chromogen. Tissue sections were imaged using a PerkinElmer Vectra 2 microscope (PerkinElmer, Waltham, MA, USA) and analyzed with PerkinElmer Inform 2.1 analysis software (PerkinElmer, Waltham, MA, USA) at MD Anderson Cancer Center Flow Cytometry & Cellular Imaging Core Facility.

2.3 Results

Adapted from previously published work by Wang et al., ***Hsp90 Inhibitor Ganetespib Sensitizes Non–Small Cell Lung Cancer to Radiation but Has Variable Effects with Chemoradiation,*** *Clinical Cancer Research, 2016, doi: 10.1158/1078-0432.CCR-15-2190.* ***Copyright at American Association of Cancer Research****.*

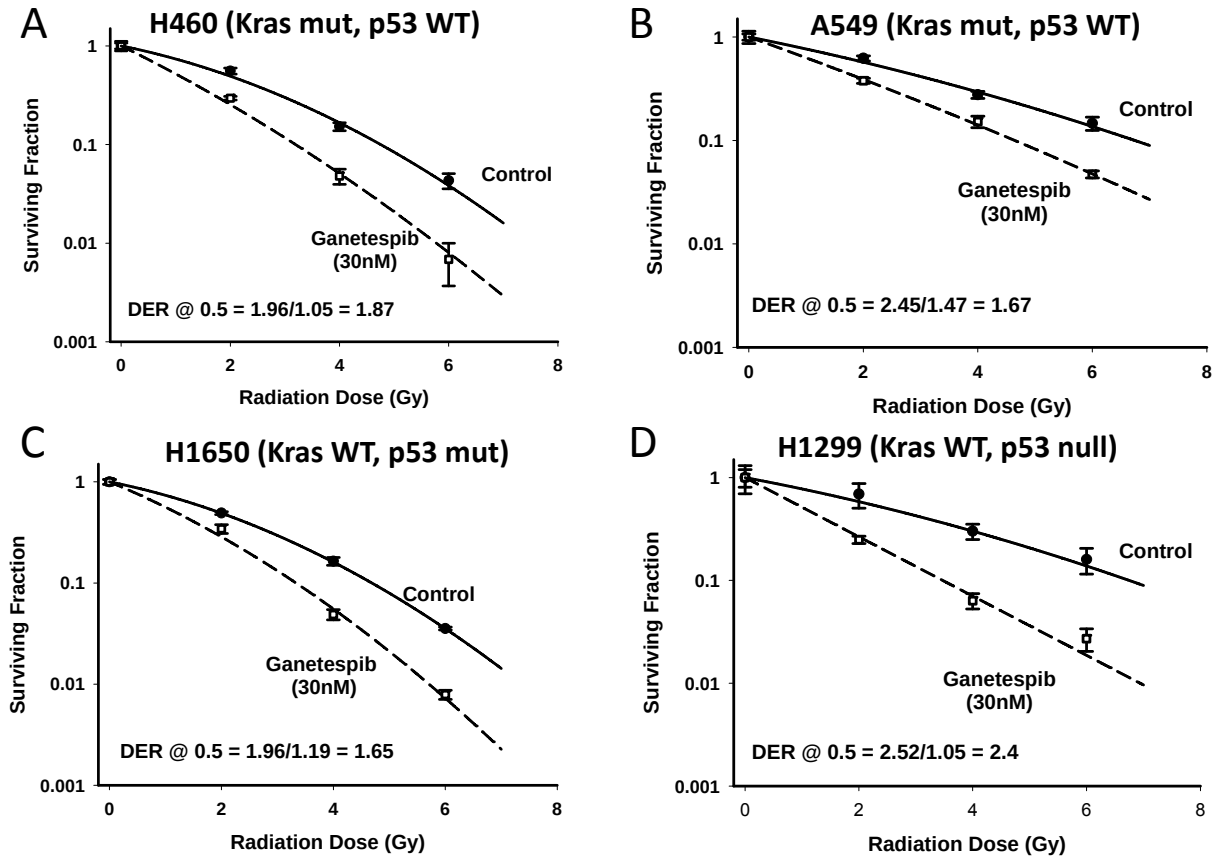
2.3.1 Ganetespib sensitizes NSCLC cells to radiation.

(The experiments in this part were mainly done by Dr. Hui Liu. Use with permission)

The ability of ganetespib to radiosensitize human NSCLC cells of varying genetic backgrounds was assessed using clonogenic survival curve assays. The Kras mutant/p53 wild type cells H460 and A549, Kras wild type/p53 mutant cells H1650 cells, and the Kras wild type/p53 null H1299 cell lines were evaluated. Cells were exposed to ganetespib at 30 nM for 5 hours, subsequently irradiated with gamma-rays and incubated for a further 19 hours in the presence of ganetespib. Irradiation in combination with ganetespib had a strong radiosensitizing effect on H460 and H1299 cells. Moderate sensitizing effects of ganetespib were observed for the A549 and H1650 cells (**Figure 1A-D**). The radiosensitivity enhancement ratios at a survival rate of 50% were 1.87 in the H460 cell, 1.67 in the A549, 1.65 in H1650 and 2.4 in H1299. The results

indicated that ganetespib can potentiate the radiation effect in different NSCLC cells.

Figure 1. Ganetespib is a potent radiation sensitizer in NSCLC cell lines. **(A-B)** Clonogenic survival curves for H460 and A549 (Kras mutant, p53 WT), **(C)** H1650 (Kras WT, p53 mutant), and **(D)** H1299 (Kras WT and p53 null). Cells were treated with or without 30 nmol/L ganetespib for 5 hours prior to irradiation followed by an additional 19 hours of post-irradiation incubation in ganetespib-containing medium.



2.3.2 Ganetespib inhibits radiation-induced DNA damage repair foci in NSCLC cancer cells.

(The experiments in this part were mainly done with help of Dr. Hui Liu. Use with permission)

Double-strand DNA breaks induced by radiation, if unrepaired, can lead to genomic instability and cell death. To determine if radiation induced DNA damage repair can be hampered by ganetespib, the DNA damage repair response was measured by evaluating 53BP1 foci formation (**Figures 2-3**). In both the H460 and A549 cells, a substantial rise in 53BP1 foci formation was seen after 30 minutes of 2 Gy radiation for both the IR alone group and the IR + ganetespib group, but not apparent with ganetespib alone. While this effect was sustained at 4 hours, by 24 hours, the number of foci was reduced in the IR alone group but not to the same level as the ganetespib alone group. However, in both cell lines, the number of foci in the cells exposed to both ganetespib and radiation was sustained significantly longer compared with radiation alone at both the 24 and 48 hour time points. This suggests that ganetespib impairs the repair of radiation-induced double-strand DNA breaks.

Figure 2. Ganetespib (Gane) attenuates DNA damage repair in H460 and A549 cells assessed by p53BP1 foci formation. Detection of p53BP1 foci formation was performed 30 minutes, 4, 24, and 48 hours after irradiation (IR; 2 Gy) with or without 50 nmol/L ganetespib pretreatment prior to irradiation

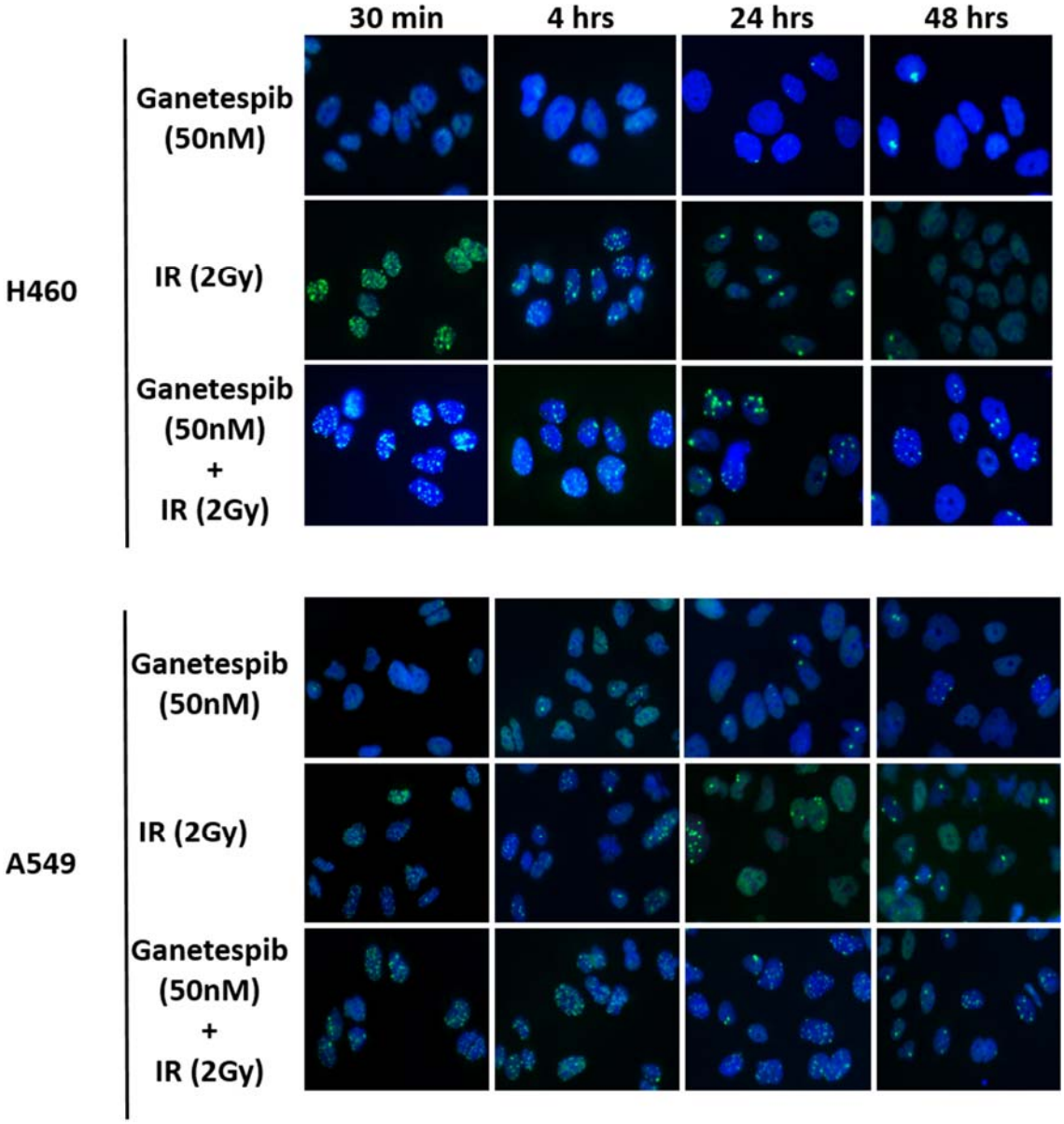
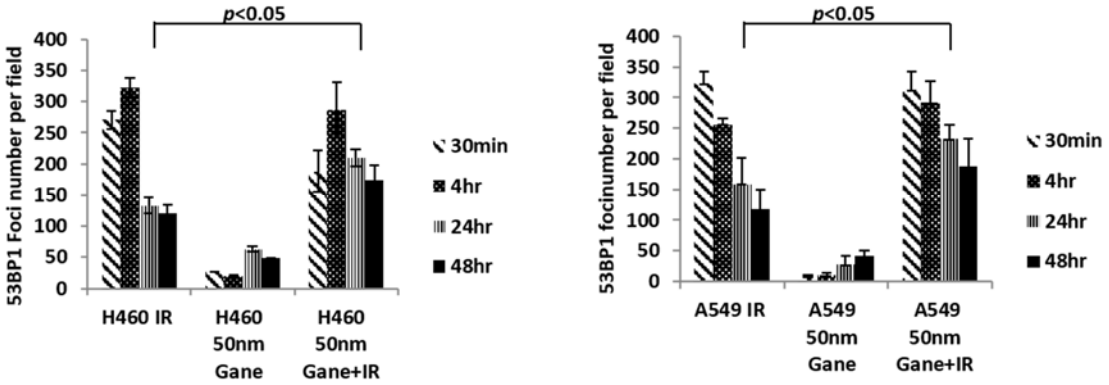


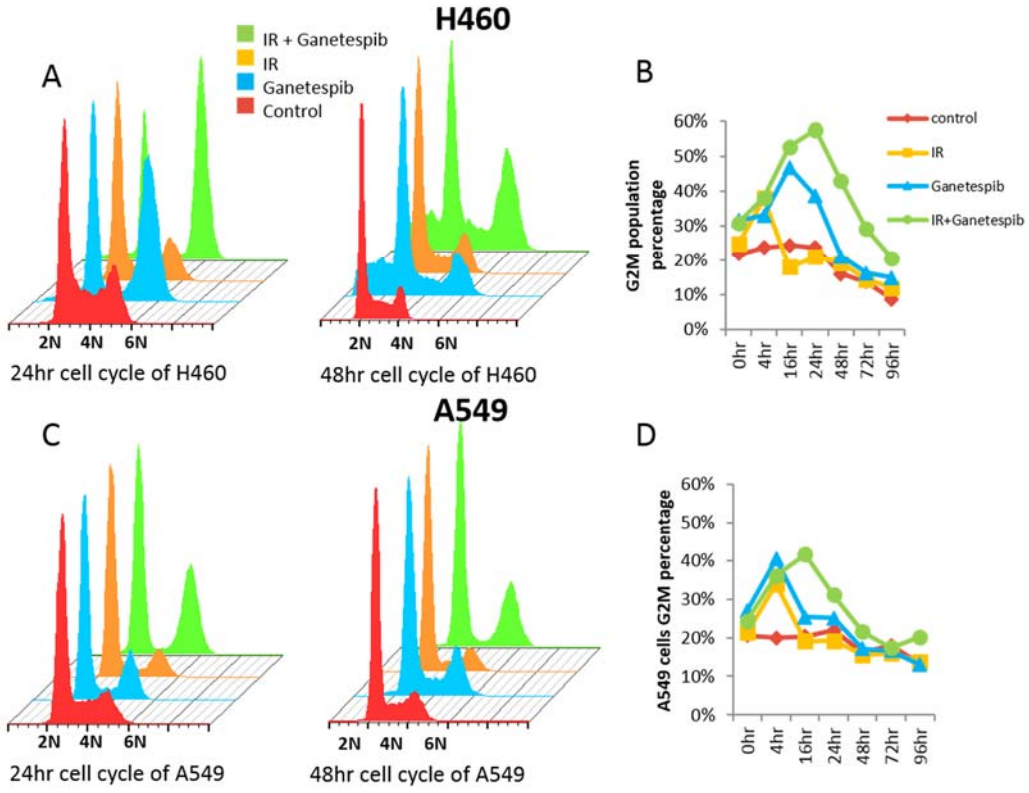
Figure 3. Statistical analysis of p53BP1 foci formation after cells were treated by radiation and ganetespib.



2.3.3 G2/M arrest induced by irradiation is further intensified by ganetespib pretreatment.

As Hsp90 inhibitors are known to affect the cell cycle checkpoint (72-77, 87), as does irradiation, which causes a G1/S arrest in P53 WT cells (88), the interaction of radiation with ganetespib on the cell cycle effects was determined in both H460 and A549 cells (**Figure 4A-D**). G2/M arrest was induced by ganestepib, radiation, and the combination, at 4 hours post-irradiation. For both H460 and A549, the irradiation effect was normalized by 16 hours, but for ganetespib alone the effect continued for 16 hours, but is mostly restored to baseline by 48 hours. The addition of ganetespib appeared to further augment the radiation-induced G2/M arrest in both H460 and A549 cells at 16-24 hour, an effect that is not fully restored to baseline in H460 until nearly 96 hours post-irradiation (**Figure 4B**).

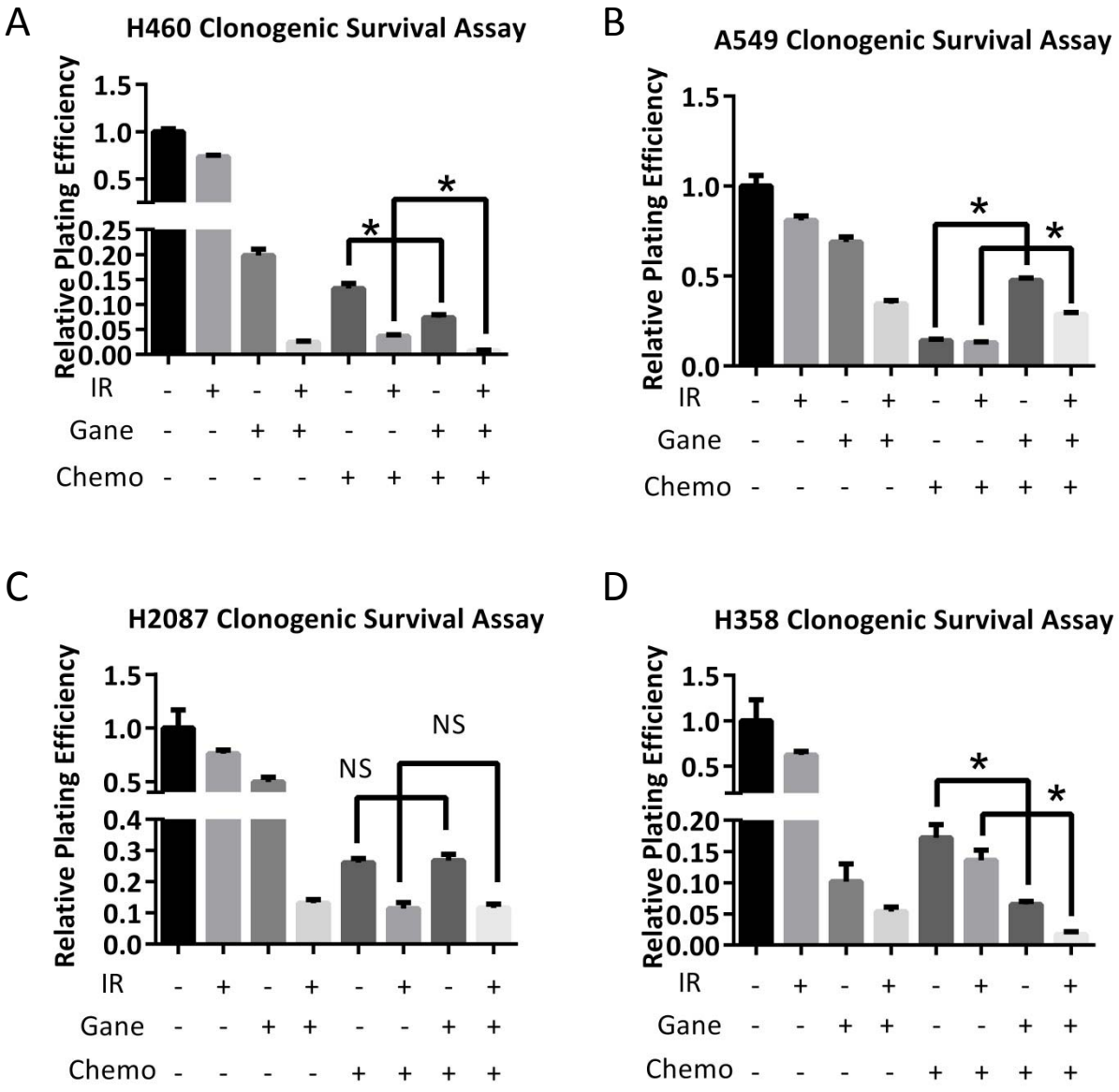
Figure 4. Ganetespib increases radiation induced G2–M arrest. H460 (**A and B**) and A549 (**C and D**) cells were treated with or without ganetespib (50 nmol/L) for 5 hours and then irradiated with 2 Gy. Cells were collected at each time point thereafter and analyzed by flow cytometry for the percentage of cells in G2–M. Drug treatment was continued after irradiation in the ganetespib-treated groups.



2.3.4 Ganetespib shows different effects with chemoradiation *in vitro*

Chemoradiation is the standard management for lung cancer. In order to assay whether ganetespib enhances the treatment efficacy of chemoradiation, clonogenic survival assay of A549 and H460 was performed (**Figure 5A-B**). The robust clonogenic survival decrease was seen in both cell lines when treated with ganetespib and IR. Unexpectedly, with the context of chemo, ganetespib enhanced treatment efficacy of chemo and chemoradiation in H460 but not in A549. In 549, ganetespib seems to protect cells from chemo shown by increased clonogenic survival of ganetespib plus chemo or chemoradiation compared with chemo or chemoradiation alone. Since A549 and H460 are both p53 wild-type cells, we tested two additional p53 mutant cell lines, H2085 and H358 (**Figure 5C-D**). Again, ganetespib sensitizes both of the cell lines to radiation, but no benefit was seen in H2085 when co-treated with chemo.

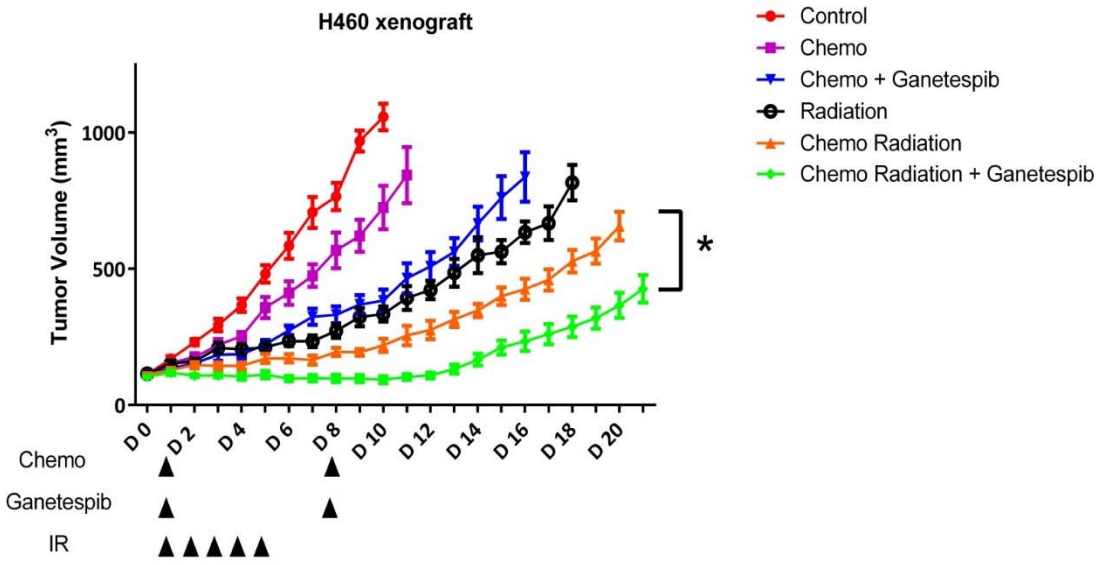
Figure 5. Ganetespib (Gane) demonstrates variable effects with CRT *in vitro*. Clonogenic survival assay results of H460, A549 (**A and B**), and H2085, H358 (**C and D**) tested with radiation alone, chemotherapy (Chemo) alone, or in combination with ganetespib.



2.3.5 Ganetespib shows different effects with chemoradiation *in vivo*

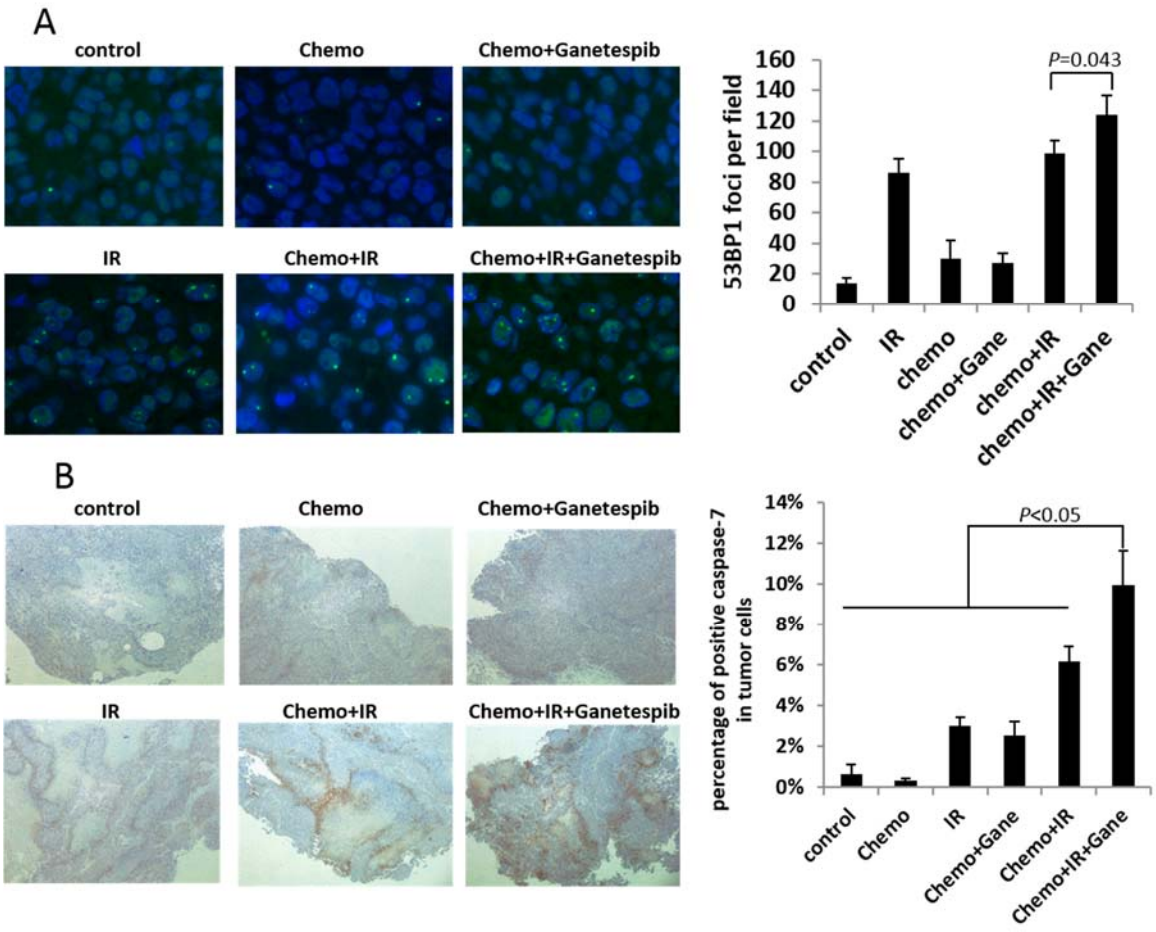
Next, the effect of ganetespib on anti-tumor treatment sensitivity *in vivo* compared to chemoradiotherapy alone was assessed. For this, a treatment protocol that reflected a shortened version of what's done clinically for unresectable NSCLC was established using fractionated daily 2 Gy radiation and concurrent chemotherapy with carboplatin and paclitaxel for 5 days, followed by consolidation chemotherapy alone for the second week. This "standard therapy" was compared to chemotherapy alone or radiation alone, with or without two doses of once-weekly ganetespib. As shown in **Figure 6**, in H460 xenografts, chemotherapy alone showed only minimal tumor growth inhibition, but when combined with ganetespib, the anti-tumor effect of chemotherapy was significantly increased. As expected, combined chemoradiotherapy had much stronger anti-tumor effect than either chemotherapy or radiation alone. However, ganetespib added to chemoradiotherapy produced the greatest tumor growth delay (**Figure 6**). Addition of ganetespib to chemoradiation delayed the tumor to reach 400 mm3 by 7 days compared with chemoradiation and delayed by 17 days compared with non-treated control.

Figure 6. Ganetespib enhanced CRT effects in H460 xenografts. Ncr nude mice were injected with 10^6 H460 cells subcutaneously in the right leg. When tumor volume reached around 100 mm^3, mice bearing established xenografts (n = 4–10/group) were exposed to radiation at 2 Gy daily for 5 days, with or without intraperitoneal 30 mg/kg carboplatin 10 mg/kg paclitaxel once per week for 2 weeks and combined with intravenous 100 mg/kg ganetespib once per week for 2 weeks, either alone or in combination. Tumor volumes are indicated as average of each treatment group and the error bars are the SEM.



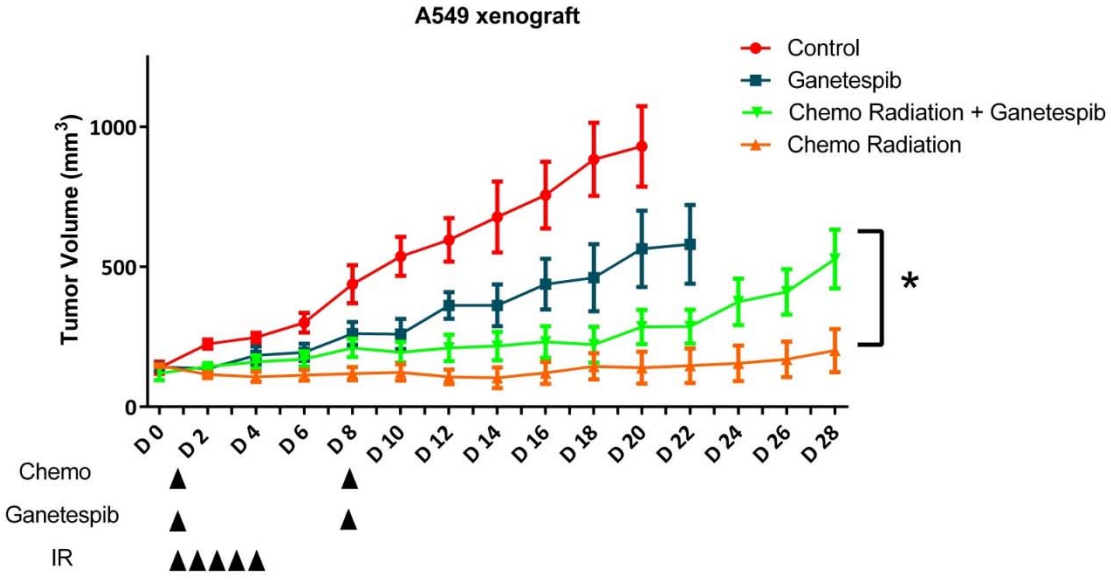
To determine if the DNA damage and cytotoxic effects of treatment in vivo could be compared to what we saw in vitro, immunohistochemical staining for 53BP1 foci and cleaved caspase 7 was performed on tumors removed at the end of the first 5 days of treatment. Compared to all the treatment groups, the tumors treated with the combination of ganetespib with chemoradiotherapy had significantly higher levels of P53BP1 foci and cleaved caspase 7 levels (**Figure 7A-B**). These results demonstrate that the enhanced DNA damage and cytotoxic effect of ganetespib with radiation seen in vitro could be further enhanced with concurrent chemoradiotherapy.

Figure 7. Ganetespib treatment increased 53BP1 foci and cleaved caspase 7 levels in H460 xenograft tumors. (A) 53BP1 foci assay. **(B)** IHC of cleaved caspase 7.



Considering the unexpected *in vitro* clonogenic survival assay results of the A549 cell line, the subsequent *in vivo* experiment was designed mainly to verify if ganetespib has no effect or even worsen the chemoradiation therapeutic effects of A549 xenografts (**Figure 8**). The data showed that ganetespib had antitumor efficiency by delaying the tumor to reach 500 mm^3 for nearly a week. Surprisingly, chemoradiation had optimal control of the tumor, but the additional treatment of ganetespib caused rapid regression after the period of tumor control. This phenomenon can be explained by increased number of clonogenic cells by ganetespib treatment in the context of chemo.

Figure 8. Ganetespib decreased CRT effects in A549 xenografts. Ncr nude mice were injected with 5 * 10^6 A549 cells subcutaneously in the right leg. When tumor volume reached around 150 mm^3, mice bearing established xenografts (n = 4–10/group) were exposed to radiation at 2 Gy daily for 5 days, with or without intraperitoneal 30 mg/kg carboplatinþ 10 mg/kg paclitaxel once per week for 2 weeks and combined with intravenous 100 mg/kg ganetespib once per week for 2 weeks, either alone or in combination. Tumor volumes are indicated as average of each treatment group and the error bars are the SEM.



2.4 Discussion

Adapted from previously published work by Wang et al., ***Hsp90 Inhibitor Ganetespib Sensitizes Non–Small Cell Lung Cancer to Radiation but Has Variable Effects with Chemoradiation,*** *Clinical Cancer Research, 2016, doi: 10.1158/1078-0432.CCR-15-2190.* ***Copyright at American Association of Cancer Research****.*

In clinical radiotherapy, tumor radioresistance is one of the causes of local failure after radiotherapy. The development of drugs that can enhance the sensitivity of tumor cells to radiation is of great importance to improve the outcomes of lung cancer therapy. Although there are many studies that have focused on the development of radiosensitizers, the targeted agents that have been tested clinically, namely vascular targeting drugs and EGFR inhibitors, have so far not been clinically useful when combined with CRT for NSCLC. Unfortunately most of rationale for the combination has been based on preclinical studies using radiation alone. The best example was the EGFR targeting drugs. It has been well established in preclinical studies that EGFR inhibition sensitizes radiation for multiple tumor types, including head and neck cancer and NSCLC (82, 89). The approach seemed promising when a survival benefit was demonstrated for cetuximab when it was combined with radiotherapy compared to radiotherapy alone in head and neck cancer in a phase III randomized trial (85). Unfortunately, the benefit of cetuximab disappeared when it was combined with CRT, as

shown in two large randomized trials for head and neck cancer (RTOG 0522 (90)) and unresectable NSCLC (RTOG 0617 (61)). This experience highlights the importance of critically evaluating radiosensitizers in preclinical models that at least modestly reflect the treatment regime used in the clinical setting.

Several studies have shown that Hsp90 inhibitors can enhance radiation sensitivity of human cancer cell lines of different origin (67-78). These sensitizing effects are the result of the Hsp90 inhibitor-mediated abrogation of the G2 checkpoint, apoptosis and the inhibition of DNA repair. Studies have shown that one of the causes of sensitization could be inhibition of DNA double strand break (DSB) repair (70-75). Checkpoint arrest mainly at G2/M phase has also been suggested as a cause of radiosensitization with Hsp90 inhibitors (72-77, 87). Radiosensitization effect in vivo by Hsp90 inhibitors has also been demonstrated (67, 74, 76). These data strongly suggest that targeting Hsp90 with its inhibitors represents a promising strategy for enhancing the sensitivity of cancer cells to radiation (72-77). Ganetespib is an investigational small molecule inhibitor of Hsp90 with favorable pharmacologic properties that distinguishes it from other first- and second-generation Hsp90 inhibitors in terms of potency, safety, and tolerability (80, 91). Ganetespib has also been shown to possess robust antitumor activity against a variety of cancer types in preclinical studies, including lung, breast, and prostate (92-97). In addition, it has been shown in NSCLC cell lines that a synergistic combinatorial benefit was seen with the taxanes such as paclitaxel or docetaxel (92).

As expected, we demonstrated that ganetespib significantly reduced clonogenic survival of various lung cancer cell lines, attenuated DNA damage repair, and induced cell cycle arrest. Since CRT with carboplatin and paclitaxel is the standard treatment regime for unresectable NSCLC, adding ganetespib to standard CRT may have synergistic effects

that need preclinical validation. Importantly, we have demonstrated that ganetespib has variable effects when combined with CRT *in vitro* and in two xenograft NSCLC models *in vivo*. Our *in vitro* clonogenic survival assay results showed that ganetespib alone enhances radiation effects in a panel of cell lines, but it demonstrated variable effects when combined with chemo or CRT. The xenograft models of H460 and A549 confirmed what we observed *in vitro*. H460 xenograft tumors gained additional benefits by adding ganetespib when treated with CRT, showing delayed tumor growth and increased DNA damage and apoptosis. On the other hand, A549 xenograft tumors was very well controlled by CRT alone but progressed rapidly when ganetespib was added. The xenograft results are consistent with what we saw *in vitro*, where there was also an increase in clonogenic cell survival when ganetespib was added to either chemotherapy or CRT. On the surface, the cell lines that do not respond to ganetespib when combined to CRT do not appear to be related to Kras or p53 mutation statuses. The mechanism for the variable effects of ganetespib in the various cell lines needs further investigation.

Our findings imply that only a subgroup of lung cancer patients may benefit from HSP90 inhibition when receiving CRT. This may warrant the need for a predictable biomarker that could potentially identify patients to receive or avoid HSP90 inhibitors in combination with chemotherapy or CRT. It is important to emphasize the fact that drugs which sensitize cancer cells to radiation may not have the same effect when added with chemotherapy or CRT. This may explain the failure of previous trials using EGFR inhibitors in combination with CRT as well as the recent futility closure of the GALAXY-II docetaxel-ganetespib trial in lung cancer. Future development of the optimal drug and radiation combination needs to be tested in rigorous preclinical models within the context of clinically-relevant therapy combinations.

Chapter 3: Radiosensitize NSCLC by MEK Inhibitor Trametinib

3.1 Introduction

Adapted from previously published work by Wang et al., ***Mutant LKB1 confers enhanced radiosensitization in combination with trametinib in KRAS-mutant non-small cell lung cancer,*** *Clinical Cancer Research, 2018, doi: 10.1158/1078-0432.CCR-18-1489.* ***Copyright at American Association of Cancer Research****.*

Lung cancer is the leading cancer type for death in the United States (4). Lung cancer is commonly managed by surgery, chemotherapy and radiotherapy. The 5-year survival rate of lung cancer is 17%, and for advanced disease, the 5-year survival is only 4% (4), indicating the huge need to improve therapeutic strategies. About 85% of lung cancer is non-small-cell lung cancer (NSCLC) and KRAS is the most frequently mutated oncogene in NSCLC (9). KRAS-mutant non-small cell lung cancer is a genetically and functionally complex disease with several subtypes, mainly defined by frequent co-mutations in the p53 and STK11 (LKB1) genes (36). These co-mutations characterize the KRAS-mutant subtype lung cancer with unique gene expression profiles and responses to chemotherapies, radiation and targeted therapies (36-38).

Radiation is a part of the standard treatments for lung cancer (6). However, KRAS-mutant lung cancers can be radioresistant (98-100). Sensitizing the tumor cells with

molecular targeted drugs can achieve higher probability of tumor control despite using the same or even lower radiation doses. In a previous study of high content radiation sensitizer screening, we identified several MEK inhibitors as radiation sensitizers for KRAS-mutant lung cancer (101). Further preclinical studies of one of the MEK inhibitors, trametinib, showed that it sensitized KRAS-mutant NSCLC cell lines to radiation *in vitro* and *in vivo* (101). Trametinib is a MEK1/2 inhibitor that has been approved for treatment of BRAF V600E mutant metastatic melanoma. Currently, trametinib is under phase I clinical trial for stage III NSCLC in combination with chemoradiation (NCT01912625). However, the effect of trametinib was highly diverse among different cell lines, thus suggests additional mechanisms contribute to the radiosensitization. It is an unmet need to identify a specific subgroup of KRAS-mutant NSCLC that can be sensitized by trametinib. The investigation of mechanisms mediating the resistance to trametinib sensitization would also enlighten novel strategies to overcome such resistance.

3.2 Materials and Methods

Adapted from previously published work by Wang et al., ***Mutant LKB1 confers enhanced radiosensitization in combination with trametinib in KRAS-mutant non-small cell lung cancer,*** *Clinical Cancer Research, 2018, doi: 10.1158/1078-0432.CCR-18-1489.* ***Copyright at American Association of Cancer Research****.*

3.2.1 Cell lines.

All the human NSCLC cell lines were obtained from American Type Culture Collection or provided by Dr. John Minna (University of Texas Southwestern Medical Center). Mouse lung adenocarcinoma cell lines were obtained from Dr. Tyler Jacks (Massachusetts Institute of Technology) or Dr. Jonathan Kurie (MD Anderson Cancer Center). All cell lines were maintained in RPMI1640 + glutamine medium supplemented with 10% fetal bovine serum in a 37 Celsius incubator with 5% carbon dioxide. Cell line authentication was carried out every 6 months of use at the Characterized Cell Line Core Facility at MD Anderson Cancer Center using the short tandem repeat method. All cell lines were regularly tested for mycoplasma.

3.2.2 Reagents and Irradiator.

Trametinib, AICAR (Acadesine) and HCQ (Hydroxychloroquine Sulfate) were purchased from Selleck Chemicals (Houston, TX). Irradiation for cells and mice were carried out

using a Shepherd mark I-68A Cesium irradiator. Dosimetry and experiment set-up were performed as we have previously described (1).

3.3.3 CRISPR knockout of p53 and Lkb1.

The lentiCRISPRv2 plasmid was provided by Dr. Feng Zhang (Massachusetts Institute of Technology) through Addgene (#52961). Guide DNA sequences were synthesized by Integrated DNA Technologies (Coralville, IA) and cloned to the plasmid according to the methods described before (102). Oligo sequences were listed in **table 1**. The constructed lentiCRISPRv2, pRSV-Rev, pMDLg/pRRE and pMD2G plasmids were transfected to 293FT cells by TransIT-LT1 (Mirus, Madison, WI) to generate lentivirus. The media of 293FT was changed after 24h of transfection and the 48h supernatant was used to infect target cells. Single cell derived stable clones were obtained after Puromycin (2~5 μg/ μL) selection for 2~3 weeks. Sanger sequencing of the targeted DNA region and western blot of targeted protein were carried out to validate the successful knockout.

Table 1. Oligo sequences for pLentiCRISPRv2 construction:

sg_m_Lkb1_F: CACCGACTCCGAGACCTTATGCCGC

sg_m_Lkb1_R: AAACGCGGCATAAGGTCTCGGAGTC

sg_m_p53_F: CACCGTGTAATAGCTCCTGCATGG

sg_m_p53_R: AAACCCATGCAGGAGCTATTACAC

sg_h_p53_1_F: CACCGACTTCCTGAAAACAACGTTC

sg_h_p53_1_R: AAACGAACGTTGTTTTCAGGAAGTC

sg_h_p53_2_F: CACCGCTTACCAGAACGTTGTTTTC

sg_h_p53_2_R: AAACGAAAACAACGTTCTGGTAAGC

sg_h_p53_3_F: CACCGCATGTGTAACAGTTCCTGCA

sg_h_p53_3_R: AAACTGCAGGAACTGTTACACATGC

sg_h_p53_4_F: CACCGCCGGTTCATGCCGCCCATGC

sg_h_p53_4_R: AAACGCATGGGCGGCATGAACCGGC

sg_Scrambled_1_F: CACCGACGGAGGCTAAGCGTCGCAA

sg_Scrambled_1_R: AAACTTGCGACGCTTAGCCTCCGTC

sg_Scrambled_2_F: CACCGCGCTTCCGCGGCCCGTTCAA

sg_Scrambled_2_R: AAACTTGAACGGGCCGCGGAAGCGC

sg_Scrambled_3_F: CACCGATCGTTTCCGCTTAACGGCG

sg_Scrambled_3_R: AAACCGCCGTTAAGCGGAAACGATC

3.2.4 siRNA knockdown.

The siRNA knockdown experiments were performed as we previously reported (45). siRNAs were transfected to cells 24h before treatment (radiation and/or trametinib). Cell lysates were collected 24h after treatment. Western blot was carried out to examine the knockdown efficiency. siRNAs used in the study were as follows: SignalSilence Atg5 siRNA I (Cell Signaling #6345), SignalSilence Control siRNA (unconjugated) (Cell Signaling #6568) and AMPK α1 siRNA (m) (Santa Cruz sc-29674).

3.2.6 LKB1 overexpression.

The whole length LKB1 was cloned by polymerase chain reaction from human cDNA, and then ligated to SFB (S-protein/Flag/SBP)-tagged plasmid driven by CMV promoter. The transfection was done by using the TransIT-LT1 (Mirus, Madison, WI) according to manufacturer's instructions. After 48h of transfection, the cells were treated by trametinib followed by radiation after 4h or single treatment alone. The transfection efficiency was examined by western blot.

3.2.6 Clonogenic survival assay.

The clonogenic survival assay was carried out as we previously described (1, 45). Briefly, cells were trypsinized to single cell suspension then seeded in 6-well plates 16 hour before treatment. Each treatment condition had 3 -6 replicates. After treated by vehicle (DMSO) or trametinib (10 – 60 nM) for 4 – 5 hours, cells were irradiated by 0, 2, 4, or 6 Gy of radiation. Drugs were washed out after 24h and cells were maintained for 2 – 3 weeks. The clonogenic survival assay for chemoradiaton was carried out as we

previously reported (1). Chemo agents (7.02 nM paclitaxel and 48.46 nM carboplatin) were mixed and were used to treat cells together with trametinib, followed by radiation after 4 hours. Media was changed after 24 hours. After the colonies were fixed and stained by 0.1% crystal violet in 20% methanol, number of colonies with at least 50 cells were counted. The data was analyzed by SigmaPlot 10.0 (Systat Software Inc.) or GraphPad Prism 7.0 (GraphPad Software, Inc). The dose enhancement ratio 0.5 (DER 0.5) was calculated as follows: DER 0.5 = (Radiation dose correlated with 50% survival fraction in DMSO treated cells)/(Radiation dose correlated with 50% survival fraction in drug treated cells). A drug of drug combination generated DER 0.5 ≥ 1.2 was considered radiosensitization.

3.2.7 Cell proliferation assay.

LKR13-scrambled and LKR13-Lkb1-KO cells were seeded in 6-well plates at a density of 5*10^4 cells per well. Cells were trypsinized and counted by a hemocytometer at day 1, 2 and 3. Three wells of cells were counted each time point for each cell type. The relative cell numbers were calculated and plotted using GraphPad Prism 7.0 (GraphPad Software, Inc).

3.2.8 Senescence associated beta-gal staining.

The senescence cells staining kit was purchased from Sigma (CS0030, St. Louis, MO). Briefly, cells were plated in 6-well plates one day prior to treatment. The next day, cells were treated by MEK inhibitor and radiation 4~5 hours later. After incubation of 72 hours, cells were fixed and stained according to the protocol provided by the manufacturer. Five

to six pictures at random fields of stained cells were taken by an inverted microscope. Then the stained blue cells (senescence positive) and non-staining cells in each picture were counted on a computer. The positive percentages were calculated and presented by GraphPad Prism 7.0 (GraphPad Software, Inc).

3.2.9 Western blots.

Western blots were carried out as we have previously described (1). Antibodies used in this study were LC3B (CST, 3868 for human, Abcam, 51520 for mouse), GAPDH (CST, 2118), HSP90 (CST, 4874), Actin (Sigma, A2228), LKB1 (CST, 3047), p53 (CST, 2524 for mouse, Santa Cruz, 47698 for human), phospho-p53-Ser15 (CST, 9284), p21 (Abcam, 109199), AMPK (CST, 5832), phospho-AMPK-Thr172 (CST, 2535), ULK1 (CST, 8054), phospho-ULK1-Ser555 (CST, 5869), ERK1/2 (CST, 4695), phospho-ERK1/2 (CST, 4370), ATG5 (CST, 8540), MDM2 (CST, 86934) and phospho-MDM2-Ser166 (CST, 3521). Quantification of band intensity was carried out by the volume tools within the Image Lab software (Bio-Rad).

3.2.10 Animal models.

Experimental methods were approved by the Institutional Animal Care and Use Committee of University of Texas MD Anderson Cancer Center. All animal experiments were carried out in accordance with the institutional guidelines and the approved protocol. Male 129S background mice were purchased from the Jackson Laboratory (Bar Harbor, ME). When the animals were 8-10 weeks old, 5*10^5 LKR13 or LKR13-Lkb1 knockout cells were subcutaneously injected to the right thigh of mice. When the

tumor diameter reached 10 mm, the mice were randomized to the following treatment arms: control, chemoradiation, Trametinib, chemoradiation + Trametinib, HCQ, and HCQ + chemoradiation + Trametinib. Chemotherapy (10 mg/kg paclitaxel and 30 mg/kg carboplatin) was administered on day 1 by intraperitoneal injection. Daily 2 Gy radiation was given for 5 days using a jig to confine animals and expose tumor to radiation as we previously reported (1). Trametinib was administered daily at a dose of 2 mg/kg for 5 days by oral gavage. Trametinib was dissolved in DMSO and 1:9 diluted in 1% carboxymethylcellulose (medium viscosity) and 0.4% Tween-80 (Sigma). HCQ was dissolved in phosphate buffered saline and administered at a daily dose of 60 mg/kg for 5 days by intraperitoneal injection. The tumor diameters were measured 2-3 times each week. The tumor volume was calculated as the following equation: Volume = 0.5 * length * width * width.

3.2.11 Assessment of lung metastasis.

Three animals from each treatment group were sacrificed at day 7 of the experiment. Lungs were fixed in 10% formalin overnight and embedded in paraffin. Three sections representing different locations from each sample were stained by Hematoxylin and Eosin. Total number of metastatic nodules from each section were counted under a microscope.

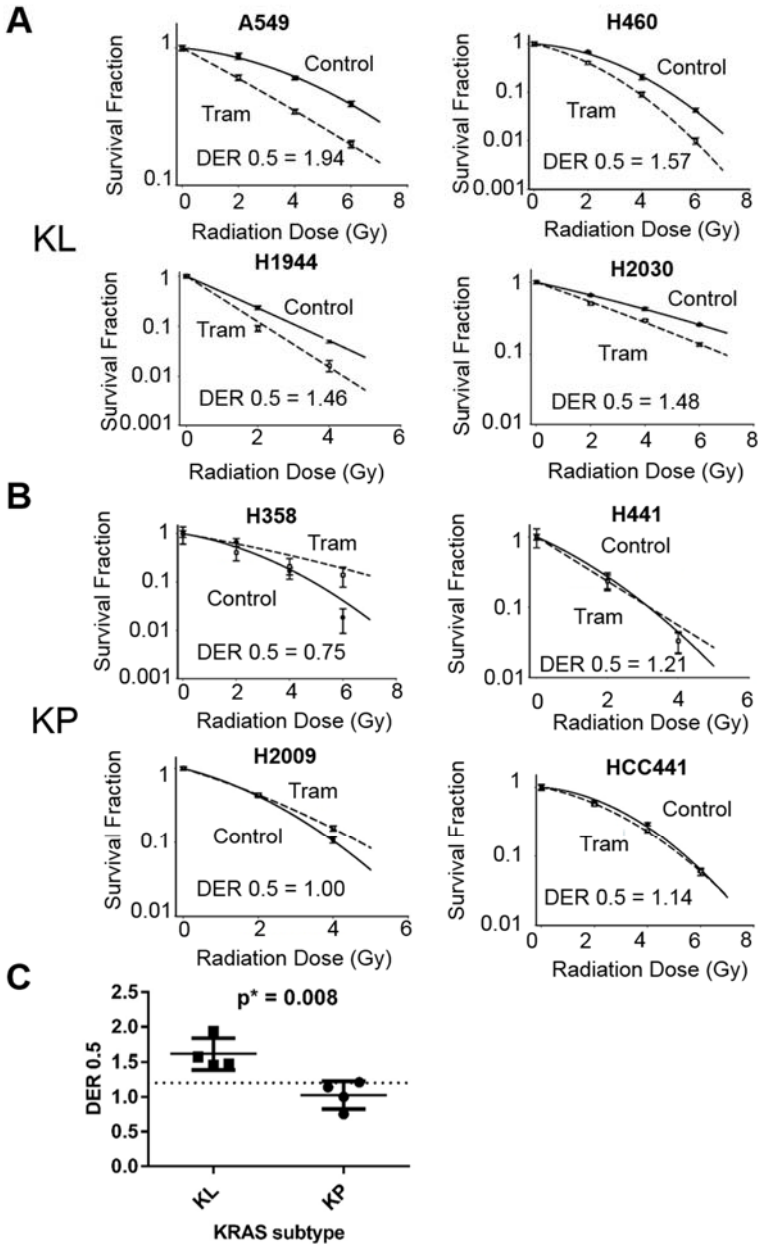
3.3 Results

Adapted from previously published work by Wang et al., ***Mutant LKB1 confers enhanced radiosensitization in combination with trametinib in KRAS-mutant non-small cell lung cancer,*** *Clinical Cancer Research, 2018, doi: 10.1158/1078-0432.CCR-18-1489.* ***Copyright at American Association of Cancer Research****.*

3.3.1 Trametinib radio sensitized KL subtype of NSCLC cell lines.

Since co-mutations of KRAS may affect cells' response to therapies, a panel of human KRAS-TP53 (KP) and KRAS-LKB1 (KL) NSCLC cell lines was tested. The clonogenic survival assay indicated trametinib radio sensitized KL cells but not KP cells (**Figure 9A-B**). The dose enhanced ratio 0.5 (DER 0.5) was used to quantify sensitization effects, and a value higher than 1.2 was usually considered sensitization. As shown in **Figure 9C**, the radiosensitization effect of trametinib was significantly higher in KL cells compared with KP.

Figure 9. The MEK inhibitor trametinib radiosensitized KRAS-LKB1 mutant non-small cell lung cancer (NSCLC) cells. (A) Clonogenic survival assays of KRAS-LKB1 (KL) NSCLC cells with or without trametinib (30 nM). DER, dose enhancement ratio. **(B)** Clonogenic survival assays of KRAS-p53 (KP) NSCLC cells with or without trametinib. **(C)** Statistical comparison (*t* test) of trametinib DER in KL and KP cells. A $DER_{0.5}$ >1.2 was used as the cut point to determine sensitization. Error bars show SEM.



3.3.2 Trametinib and radiation induced p53-dependent senescence in KRAS-LKB1 mutant NSCLC cell lines.

Next, the mechanism of trametinib induced radio sensitization was investigated. The KL cell H460 displayed enlarged and flat morphology after the combination treatment, suggesting senescence might be up-regulated (**Figure 10A**). Such morphology change was not observed in KP cell H441 (**Figure 10B**). Next, the senescence marker, beta galactosidase, was detected by beta-gal staining and the results indicated that trametinib and radiation induced strong senescence in the KL cell H460 but not in KP cell H441 (**Figure 10C-D**). The senescence marker, p21, was up-regulated in KL cells A549 and H460 after 48h of treatment, but not in the KP cell H441. The p21 level in A549 and H460 after treated by combination of radiation and trametinib was higher than single treatment alone, suggesting the combination may robustly induce senescence (**Figure 11A**). To understand if p53 was required to induce senescence by the combination treatment, p53 knockout A549 and H460 cell lines were generated by CRISPR-Cas9 targeting. The knockout cells did not express p53 and were not able to up-regulate p21 after radiation induction (**Figure 11B**). Clonogenic survival showed p53 knockout cells were not radio sensitized by trametinib (**Figure 11C**) while senescence was absent in the p53 knockout cells (**Figure 11D**). Next, we examined the level of MDM2 in A549 and H460 after treatments by western blot. As expected, MDM2 expression was up-regulated by radiation. Trametinib did not change MDM2 expression level, but robustly inhibited MDM2 phosphorylation at Ser-166, which was reported before to increase the binding affinity of MDM2 with p53. As a result, p21 was strongly activated only by the combination of radiation and trametinib (**Figure 12**).

Figure 10. Trametinib and radiation induced senescence in KL cells. (A-B) Combined trametinib plus radiation led to enlarged cells in the *KRAS-LKB1*-mutated (KL) cell line H460 but not in the *KRAS-p53*-mutated (KP) cell line H441. **(C-D)** Senescence-associated (SA) -beta gal staining results of H460 (KL) and H441 (KP) cells. T-test was used to compare the difference between groups. Error bars show SEM.

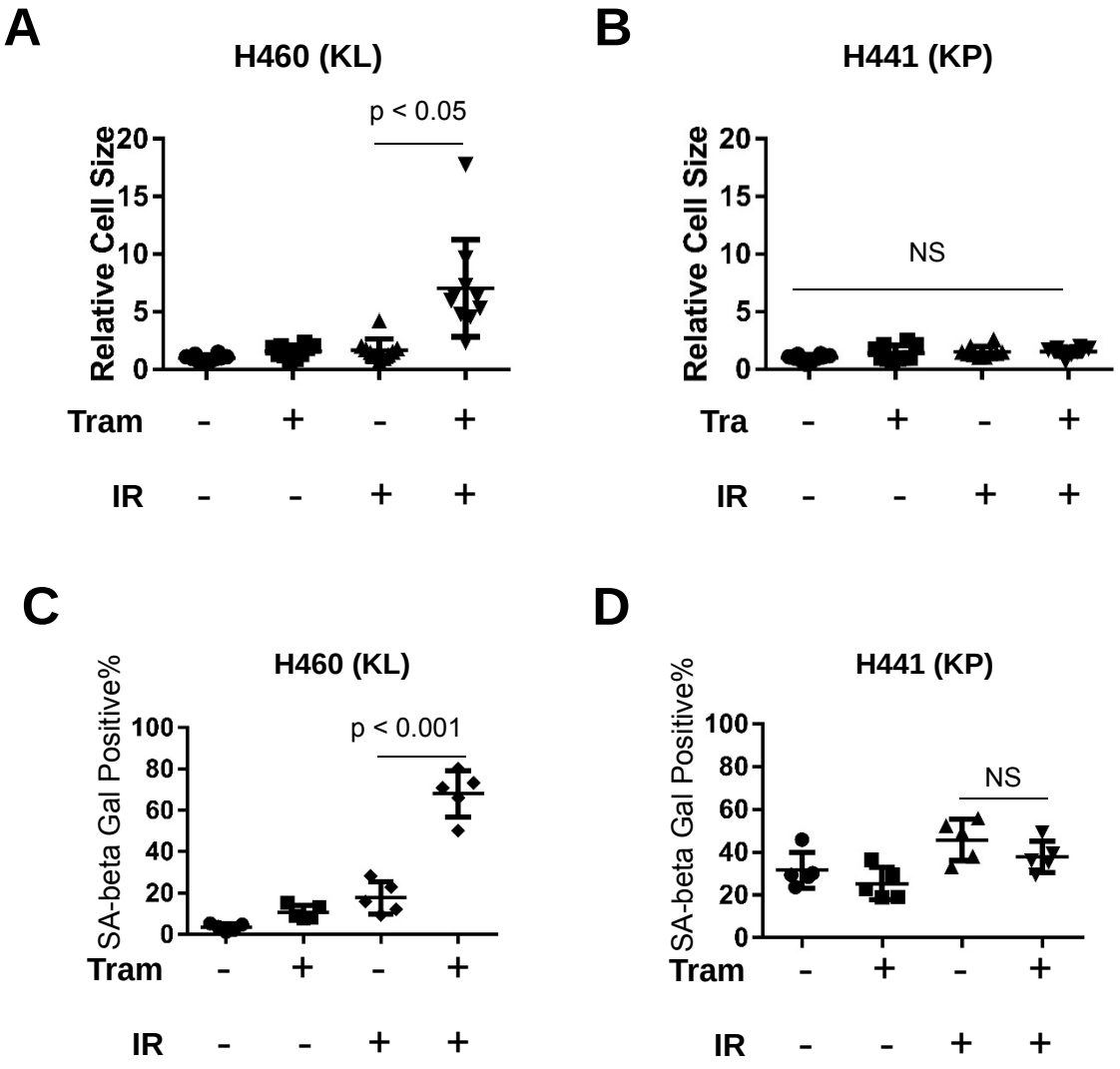


Figure 11. Trametinib and radiation induced senescence was p53-dependent. (A) Western blot of senescence marker, p21, after trametinib (30 nM) and radiation (2 Gy) treatments in A549, H460 and H441 cells. **(B)** Western blot of p53 and p21 in p53 knockout and scrambled control cells treated or not treated with 6 Gy of ionizing radiation. Tram, trametinib. **(C)** Clonogenic survival assays of p53-knockout A549 and H460 cells.

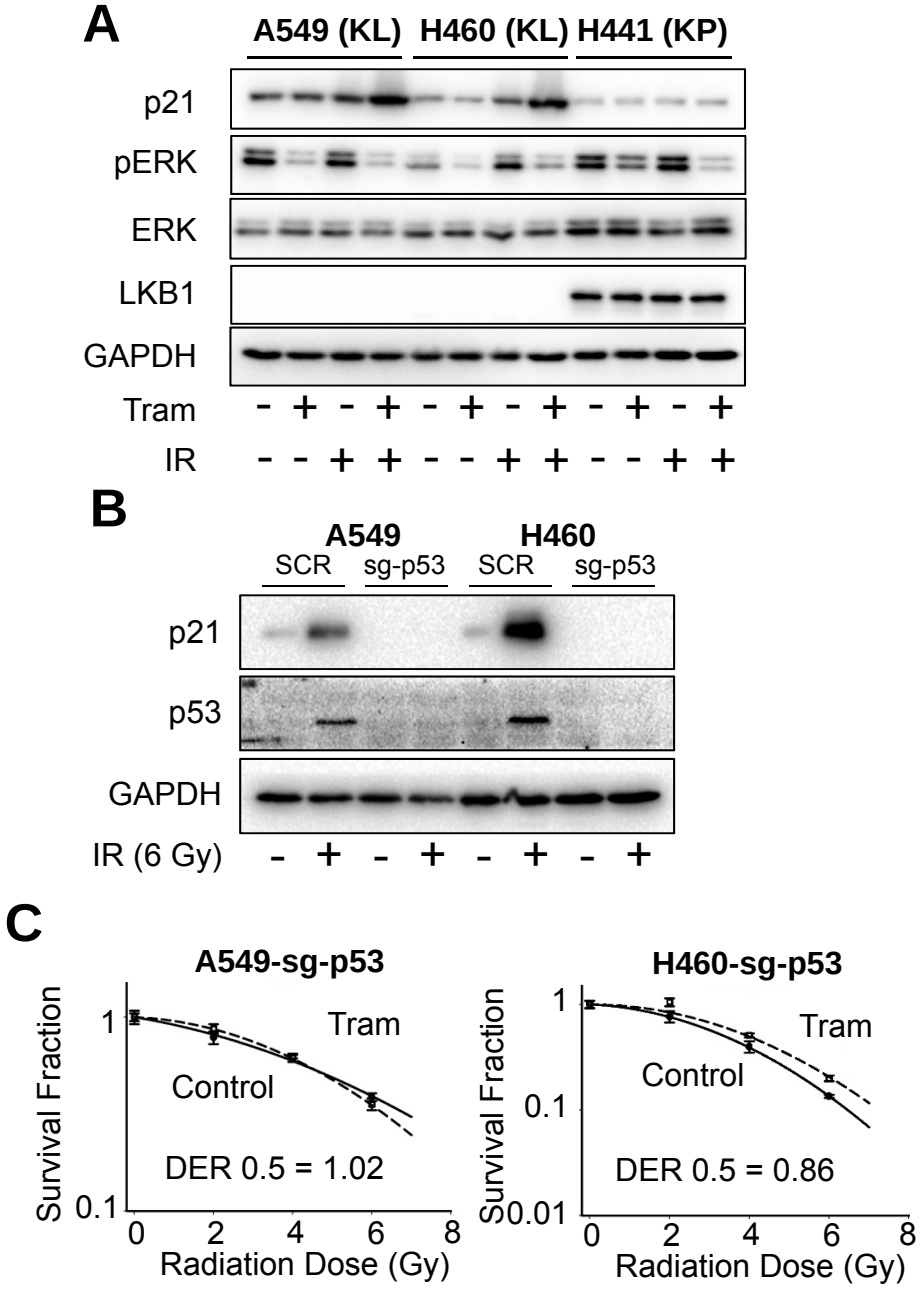


Figure 11 (Continued). (D) Senescence-associated beta galactosidase staining of scrambled control and p53-knockout H460 cells.

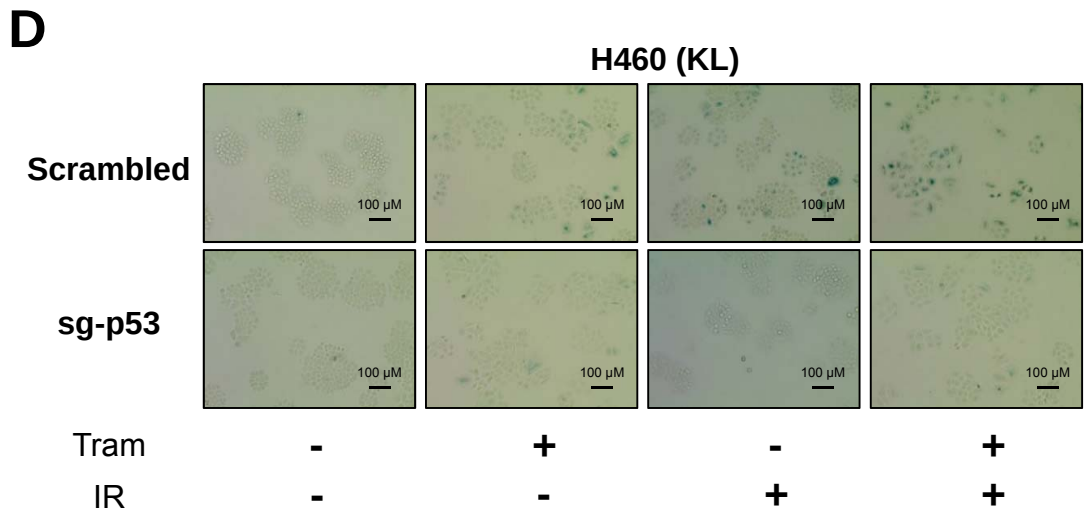
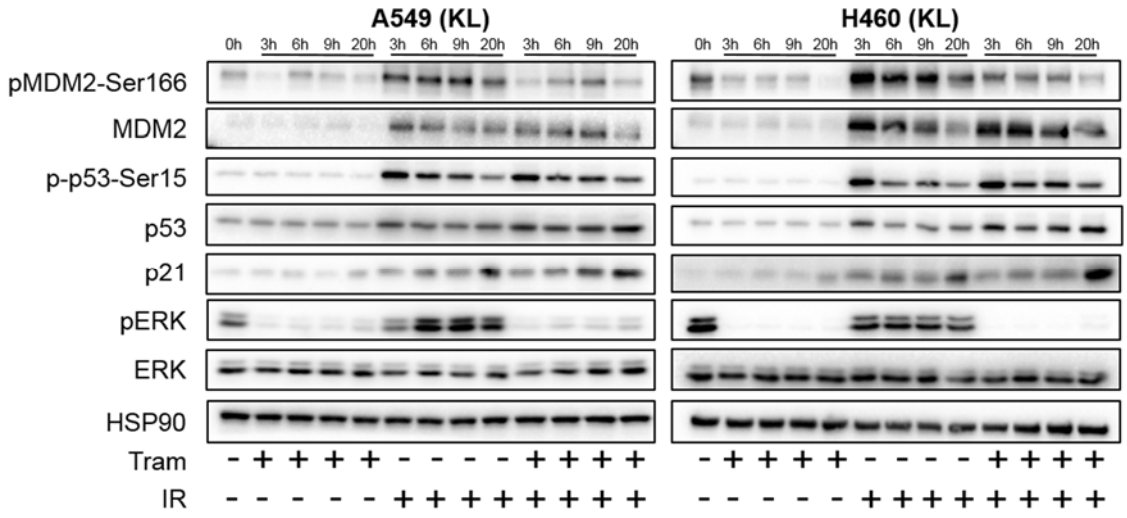


Figure 12. Trametinib increased MDM2-Ser166 phosphorylation to stabilize p53. Western blot of MDM2 Ser166 level changes in A549 and H460 at different times after treatments.



3.3.3 LKB1 abrogated radio sensitization effect of Trametinib.

To better understand the functions of co-mutations (TP53 and LKB1) in KRAS-mutant lung cancer, a Kras MUT, p53 and Lkb1 WT mouse lung cancer cell, LKR13, was used to generate p53 and Lkb1 knockout cells (**Figure 13A-B**). With the KP and KL cells, their response to radiation and trametinib alone was firstly tested. The p53 and Lkb1 knockout in LKR13 induced radio resistance (**Figure 13C**). Interesting, Lkb1 loss also increased the resistance to trametinib (**Figure 13D**). Next, the cells response to trametinib induced sensitization was tested. Similar as in human lung cancer cell lines, trametinib radio sensitized the Lkb1 knockout cells in a dose-dependent manner, but not the scrambled control or the p53 knockout cells (**Figure 13E**). In order to investigate the role of LKB1 in trametinib induced radiosensitization, overexpressed of LKB1 was performed in A549 and H460. Clonogenic survival assay showed that the A549-LKB1 and H460-LKB1 was resistant to trametinib sensitization (**Figure 13F-G**). The overexpression of LKB1 in A549 decreased senescence after combination treatment (**Figure 13H**).

Figure 13. LKB1 loss of function was required for trametinib-induced radiosensitization. (A-B) Western blots of Lkb1 and p53 in several stable clones. **(C-D)** Trametinib IC50 and survival fraction at 2 Gy of LKR13 scrambled control, p53 knockout, and Lkb1 knockout clones. Three stables clones from each knockout and scrambled control were tested.

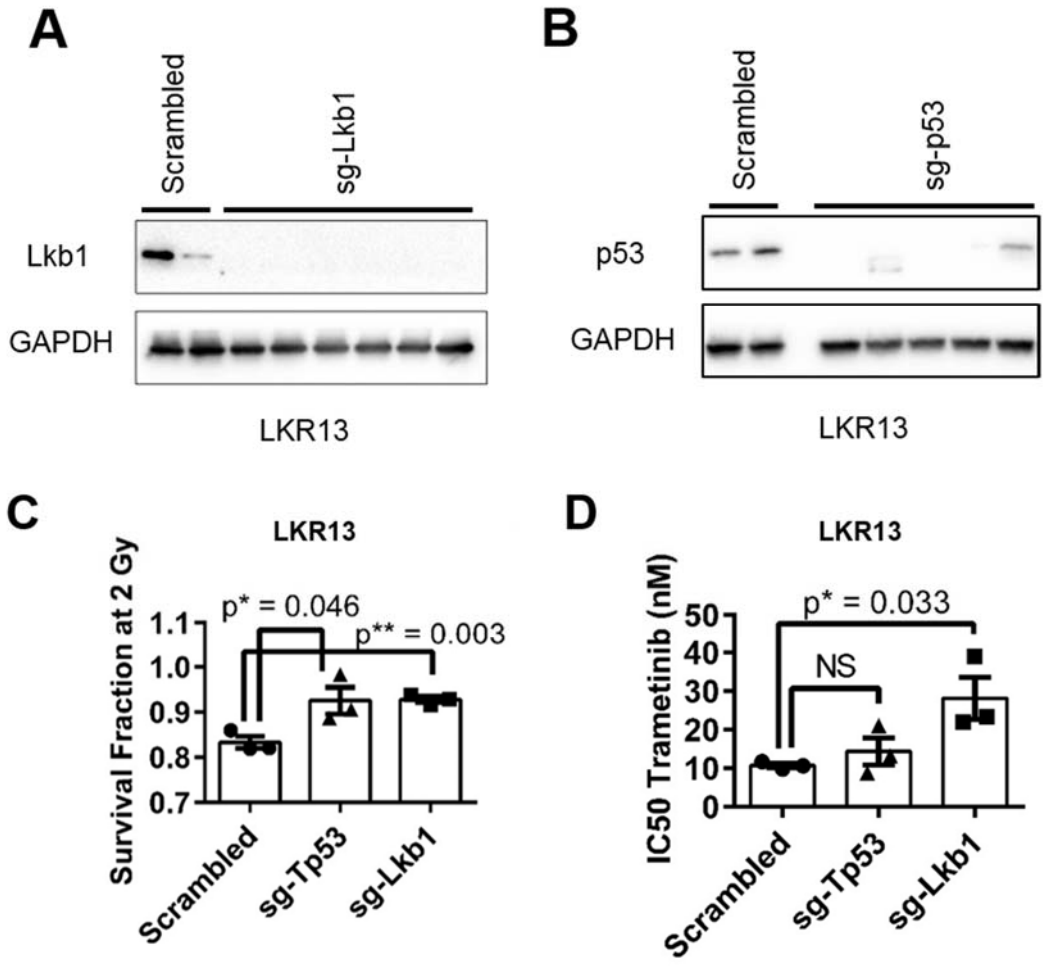
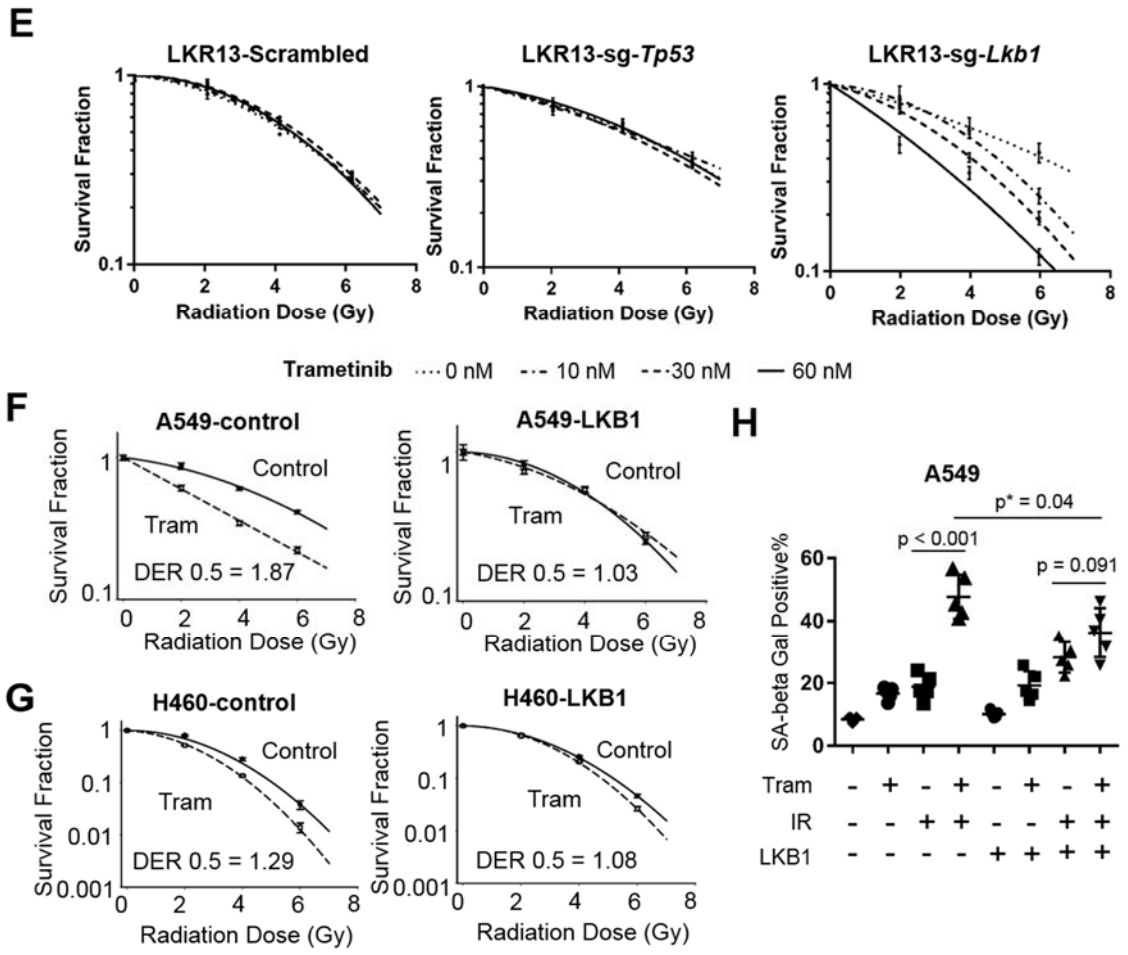


Figure 13 (Continued). (E) Clonogenic survival assays of LKR13 knockout cells with different concentrations of trametinib. **(F-G)** Clonogenic survival assays of A549 and H460 cells with LKB1 overexpression treated by trametinib (30 nM) and radiation. **(H)** SA-beta gal staining results of A549 control and LKB1- overexpressing cells.



3.3.4 Resistance to trametinib radiosensitization may be rendered by AMPK pathway through LKB1.

The mechanism of LKB1 induced resistance to trametinib sensitization was investigated as the next step of the study. One of the pathways that was directly regulated by LKB1 was AMPK. Western blot of LKB1 overexpressing A549 cells, and LKR13 knockout cells after 24h of combination treatment showed AMPK was robustly activated in the cells with WT LKB1 (**Figure 14A-B**). The combination activated stronger AMPK than single treatments alone. AMPK activation was completely absent in the A549 and LKR13 cells without LKB1, suggests the activation of AMPK by radiation and trametinib might be LKB1 dependent. In order to investigate the roles of AMPK pathway in trametinib induced radio sensitization, the A549 cells were treated with AMPK activators (AICAR) simultaneously with trametinib and radiation. AICAR was able to activate AMPK in A549 despite LKB1 loss (**Figure 14C**). In the clonogenic survival assay, activation of AMPK abrogated the radiosensitization effect of MEKi (**Figure 14D**). Next, AMPK was knocked down in LKR13 cells by siRNAs (**Figure 14E**). The LKR13-AMPK-KD cells were radio sensitized by MEKi, despite wildtype LKB1 (**Figure 14F**).

Figure 14. LKB1-induced activation of AMPK blocked trametinib-induced radiosensitization. (A-B) Western blot of phospho-AMPK and phospho-ERK in A549-LKB1 cells and LKR13 p53- or LKB1-knockout cells after trametinib (30 nM) with radiation (2 Gy for A549, 4 Gy for LKR13). **(C)** Western blot of phospho-AMPK in A549 cells at different times after treatment with the AMPK activator AICAR. **(D)** Clonogenic survival assays of A549 cells treated with trametinib (30 nM), AICAR (0.5 μM), and trametinib + AICAR.

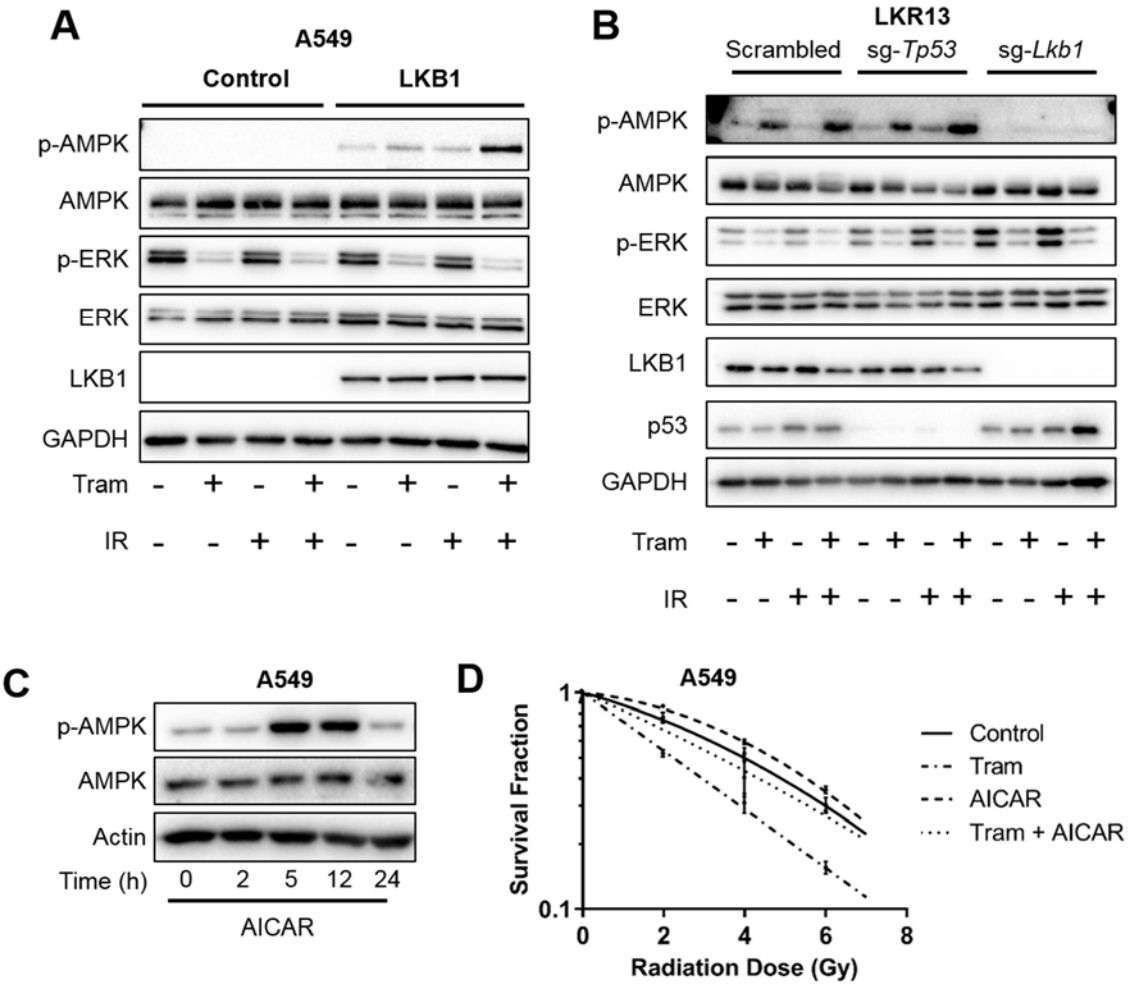
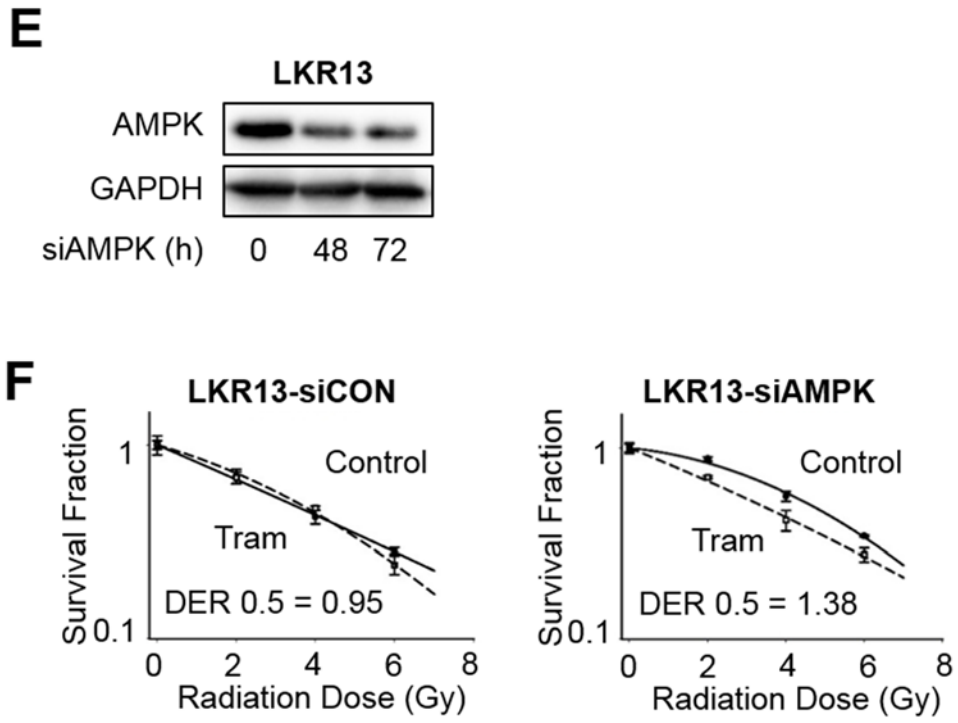


Figure 14 (Continued). (E) Western blot of AMPK in LKR13 cells transfected with AMPK siRNA. **(F)** Clonogenic survival assays of LKR13 and LKR13-AMPK-KD cells treated by trametinib.



3.3.5 Blocking of autophagy reversed resistance to trametinib sensitization.

AMPK is known to regulate autophagy (103, 104), which is a self-degradation program in starving and damaged cells (105). Some studies have indicated that autophagy may protect damaged cells from senescence (106-108). Whether the AMPK activation resulted in up-regulated level of autophagy was further examined. As we expected, the autophagy activity was up-regulated in LKB1 overexpressing A549 cells, evidenced by the increased LC3B II/I radio (**Figure 15A-B**). Since autophagy has been reported to rescue cells from senescence, whether inhibition of autophagy could increase senescence in LKB1 wildtype cells was tested. The results showed that A549-LKB1 cells undergone senescence after treated by HCQ, trametinib and radiation (**Figure 15C**). The A549-LKB1 and LKR13 cells were radio-sensitized by HCQ and trametinib but not trametinib alone (**Figure 15D-E**). Knockdown of ATG5 in A549 reversed the resistance to trametinib radio-sensitization (**Figure 15F-G**).

Figure 15. Autophagy inhibition partially potentiated LKB1 wild-type cells to trametinib-induced radiosensitization. (A-B) Western blot of autophagy marker LC3B in A549 cells treated by trametinib (30 nM) and radiation (2 Gy). Error bars show SD. **(C)** SA-beta gal staining results of A549-LKB1 cells treated by trametinib (30 nM), radiation (2 Gy), and HCQ (50 μM).

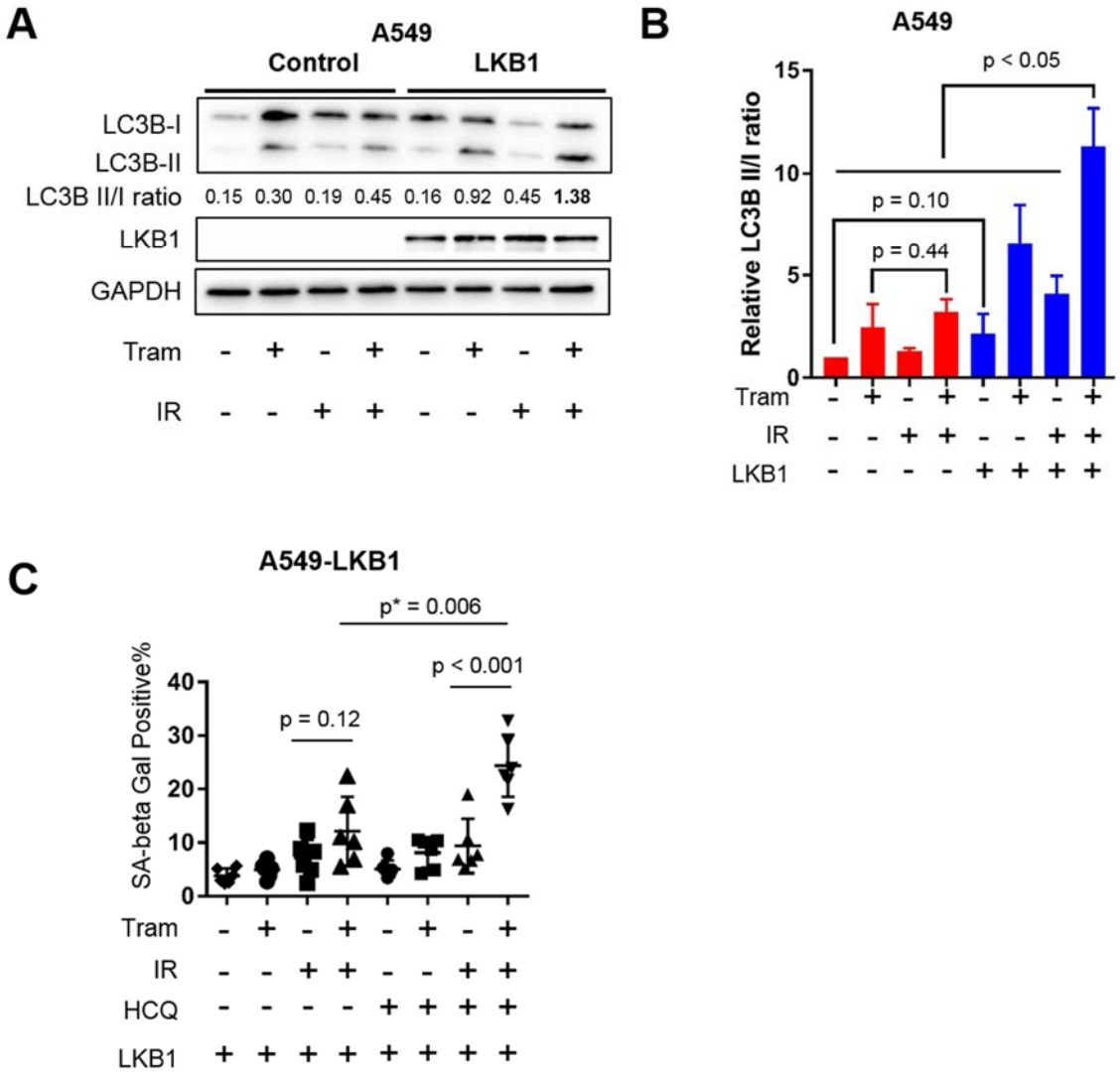
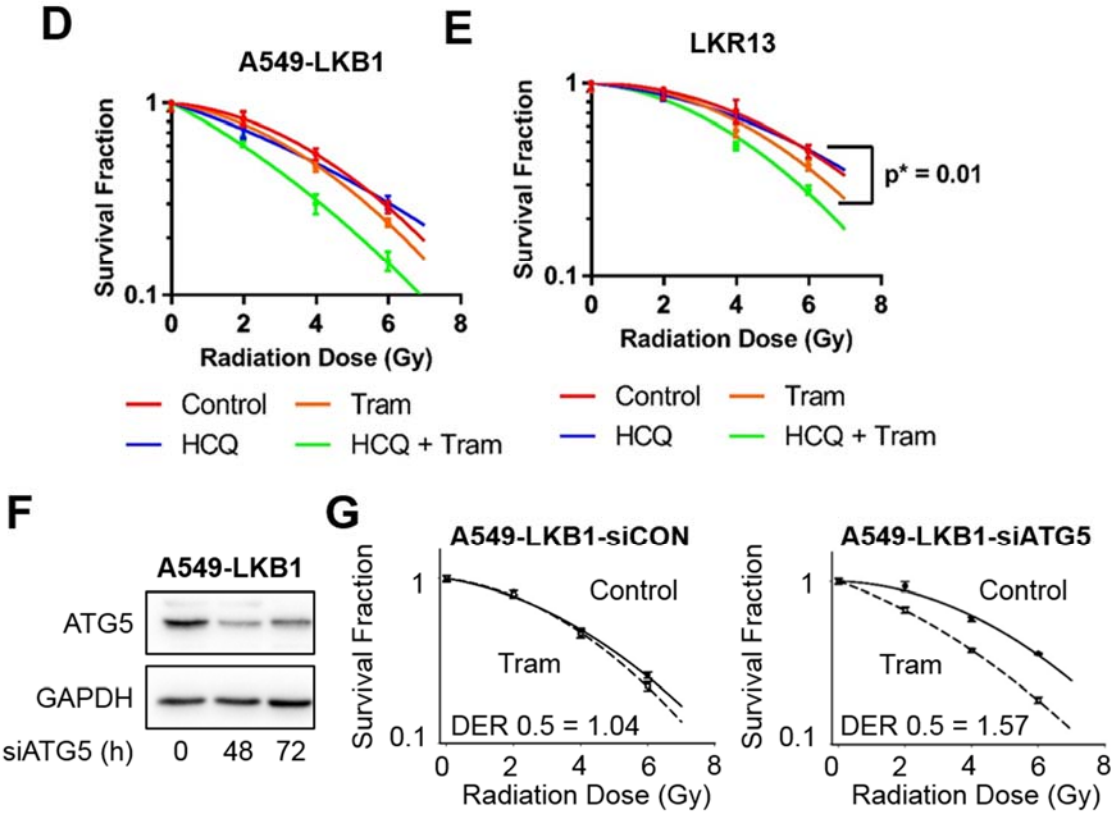


Figure 15 (Continued). (D-E) Clonogenic survival assay of A549-LKB1 and LKR13 cells treated with trametinib (30 nM) and HCQ (50 μM). **(F)** Western blots of ATG5 in A549-LKB1 cells after transfection with ATG5 siRNA. **(G)** Clonogenic survival assay of A549-LKB1-ATG5-KD and scrambled KD cells.



3.3.6 LKR13-Lkb1 knockout tumors were resistant to trametinib or chemoradiation, but were sensitive to the combination.

To validate the findings, subcutaneous tumors using the LKR13-Lkb1 knockout cell lines and the scrambled control were established. Mice were treated by chemoradiaton with a setting that mimics the treatment regime in human (**Figure 16A**). Clonogenic survival assay showed that trametinib sensitized LKR13-Lkb1 knockout cells to both radiation and chemoradiation *in vitro* (**Figure 16B**). After implanted into mice, the Lkb1 knockout tumors were much more aggressive than the scrambled control (**Figure 16C**), despite similar growth rates in vitro (**Figure 16D**). For the LKR13-Lkb1-KO tumors, the tumor growth results indicated that neither trametinib alone nor chemoradiation alone effectively inhibited the tumor growth, however, the combination of trametinib and chemoradiation achieved significantly better tumor control (**Figure 16E**). In both the control and chemoradiation-treated group, the animals had significant morbidity and died rapidly. Lung tissues were collected at day 7 and H&E staining revealed numerous metastatic nodules in the control and chemoradiation group, that were significantly reduced by trametinib, and was further reduced by chemoradiation (P = 0.04) (**Figure 6F-G**). Although trametinib treatment alone increased survival compared with control (p = 0.026), the animals were being sacrificed when primary tumor burden exceeds the limit. The combination of chemoradiation and trametinib stabilized tumor growth and achieved long-term survival (p < 0.0001) (**Figure 16H**).

Figure 16. Combining trametinib with chemoradiation synergistically inhibited Kras-Lkb1-mutant murine lung tumor growth *in vivo*. (A) Treatment schema for tumor-bearing mice. **(B)** Clonogenic survival assays of LKR13-Lkb1-KO cells after Chemo (IC_{50}), trametinib (10 nM), and both. **(C)** Volumes of LKR13-Scrambled control tumors and LKR13-Lkb1-knockout (KO) tumors 2 weeks after inoculation. **(D)** *In vitro* cell growth assay of LKR13 scrambled control and Lkb1-KO cells.

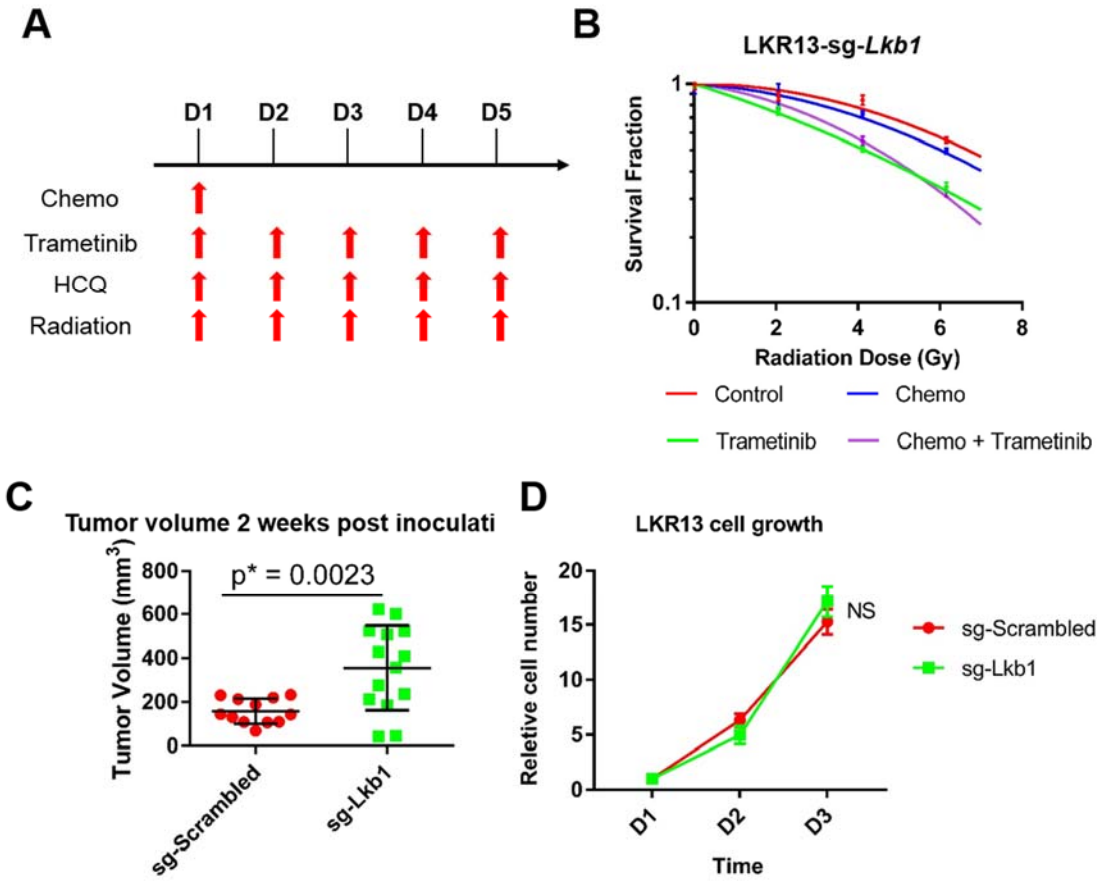
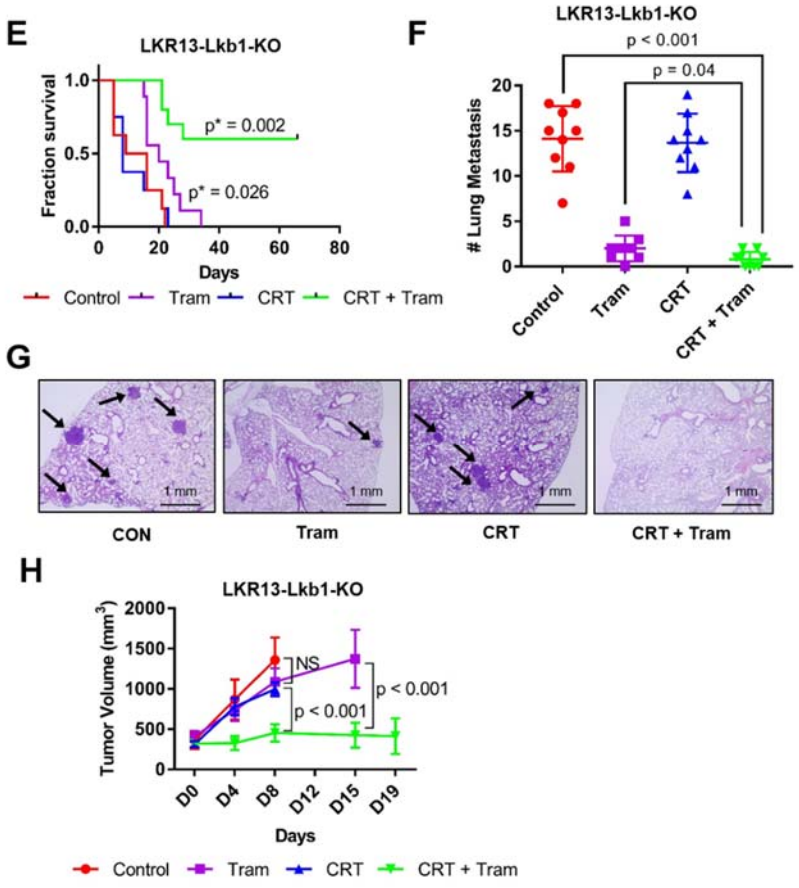
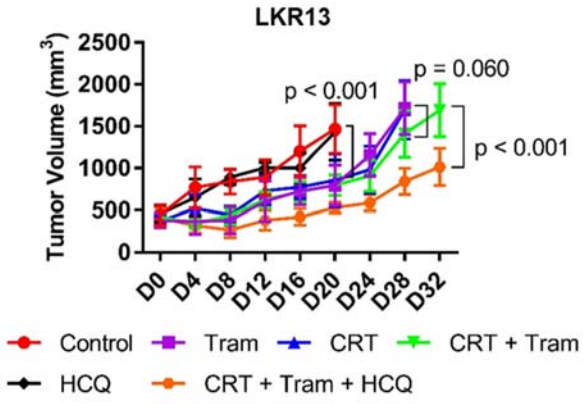


Figure 16 (Continued). (E) Growth curves of LKR13-Lkb1-KO tumors after treatment of vehicle control, trametinib, chemoradiation, and trametinib plus chemoradiation. Six to ten mice were randomized to each treatment group. Error bars show SD; *t* tests were used to compare differences in tumor volume at different times. **(F)** Number of lung metastasis nodules detected in each group. Error bar show SD; *t* tests were used to compare the difference. **(G)** Representative staining of lung sections. Arrows pointing nodules. **(H)** Survival curves of LKR13-Lkb1-KO tumor-bearing mice. Log-rank tests were used to compare difference between curves.



For the LKB13-Scrambled tumor, both trametinib alone and chemoradiation alone significantly inhibited tumor growth comparing with control. The combination of chemoradiation and trametinib trended to be better than trametinib or chemoradiation, but did not yield a statistical significance at 0.05 level ($p = 0.060$). The autophagy inhibitor HCQ alone had no effect on tumor growth, but the combination of HCQ, trametinib and chemoradiation had superior tumor growth inhibition compared with trametinib and chemoradiation or single treatment alone ($p < 0.001$) (**Figure 17**).

Figure 17. HCQ potentiated trametinib-induced chemoradiation sensitization in LKR13 tumors. Growth curves of LKR13 scrambled control tumors after treatment of vehicle control, trametinib, hydroxychloroquine (HCQ, autophagy inhibitor), chemoradiation, trametinib plus chemoradiation, and HCQ, plus trametinib plus chemoradiation. Six to ten mice were randomized to each treatment group. Error bars show SD; *t* tests were used to compare differences in tumor volume at different times.



3.4 Discussion

Adapted from previously published work by Wang et al., ***Mutant LKB1 confers enhanced radiosensitization in combination with trametinib in KRAS-mutant non-small cell lung cancer,*** *Clinical Cancer Research, 2018, doi: 10.1158/1078-0432.CCR-18-1489.* ***Copyright at American Association of Cancer Research****.*

In the current study, we demonstrate that the tumor suppressor, LKB1, could induce the resistance to combination therapies of radiation and MEK inhibitor, trametinib. In KRAS-mutant NSCLC, LKB1 could induce resistance to the combination of trametinib and radiation through activating the AMPK-autophagy pathway to rescue cells from undergoing senescence. As a result, the *KRAS-LKB1* co-mutant tumors were very sensitive to the combination, due to the absent of LKB1-driven resistant mechanism. The trametinib and radiation induced senescence in the cells with KRAS and LKB1 mutations, but not in the cells with loss-of-function p53 or with wildtype LKB1. This intriguing phenomenon could be the result of the complicated interplay between the MAPK pathway and the tumor suppressor pathways. When administered with radiation, trametinib simultaneously affected the two tumor suppressor pathways: stabilizing p53 by decreasing the MDM2-Ser166 phosphorylation and activating AMPK pathway depending on LKB1. The p53 pathway is well known to be activated by radiation (109), but the level of activation is strictly regulated by the negative feedback loop of MDM2. MDM2 phosphorylation at Ser166 was reported to increase the ubiquitin ligase activity of

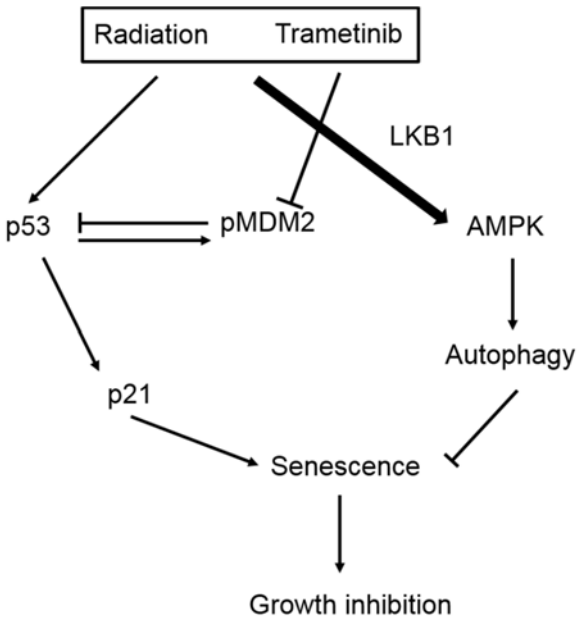
MDM2 (110, 111), thus, trametinib could stabilize the p53 protein after radiation to augment the radiation-induced senescent effects. In the LKB1 wildtype tumors, however, trametinib also synergized with radiation to induce a robust AMPK activation and eventually up-regulated autophagy to rescue cells from senescence. Autophagy is one of the known cellular mechanisms to prevent cells from undergoing senescence (106-108). In a recent study of breast cancer, it was found out that the CDK 4/6 inhibitor, Palbociclib, induced both autophagy and senescence. The inhibition of autophagy by HCQ sustained tumor cell growth and increased senescence both *in vitro* and *in vivo* (112). In summary, although LKB1 mutation rendered resistance to either radiation or trametinib alone, it makes the KL subtype of NSCLC a specific group that is vulnerable to the combination of two modalities, due to the absence of LKB1-AMPK-autophagy driven resistance pathway. The proposed working model is illustrated in **Figure 18**.

Usually, deficiencies in tumor-suppressors are linked with tumor initiation and progression. Thus, boosting or restoring tumor-suppressor signaling could be a strategy to control cancer. The inhibitors of MDM2, a p53 ubiquitase, could stabilize the p53 pathway and inhibit tumor growth in preclinical models (113-117). Several MDM2 inhibitors are under clinical investigations (118, 119). The activation of another tumor suppressor, LKB1, and its downstream AMPK pathway has been recognized as a novel cancer therapeutic strategy supported by growing evidence. The AMPK activator, metformin, has been used for diabetes treatment, but recent research has demonstrated its anti-tumor effects (120-123).

A previous study of our group showed that radio sensitization effects of ganetespib, a HSP90 inhibitor, were reversed by chemotherapy in some cell lines *in vitro* and *in vivo* (1). The findings of our present study once again emphasize the complexity of tumor

signaling depending on the genetic background, especially when treated with combined modalities. Our study also suggests the predictive biomarker for a certain treatment may not be applicable when combined with other therapies. Finally yet importantly, our findings identify *KRAS-LKB1* mutant NSCLC as a subgroup that is most likely to benefit from the combination of trametinib inhibition and chemoradiation. For the NSCLC with wildtype *LKB1*, inhibition of autophagy could a potential strategy to overcome the resistance to certain treatments. Whether this is translatable to clinic needs future clinical trials or retrospective analysis of existing clinical data.

Figure 18. Schema of proposed trametinib radiosensitization mechanism.



Chapter 4: Discussion

Sensitizing lung cancer to radiation could potentially improve the treatment efficacy using same or ever lower dose of radiation without significantly increasing the toxicity. Therefore, identifying novel radiosensitizers is a current unmet need. In my study, both the HSP90 inhibitor ganetespib and the MEK inhibitor trametinib showed radiosensitization effects in NSCLC, however their efficacy is dependent on the treatment combination or the genetic background of the cell. The results of my research have several important translational implications.

First, the results of variable effects of ganetespib suggests the need to test novel drugs and treatment combinations in a treatment context that reflects the real clinic. Cells that respond to combination with two modalities may not still benefit from additional treatments. In my study, ganetespib sensitized a panel of NSCLC cell lines to radiation. Further mechanistic studies showed that ganetespib intensified radiation induced G2/M arrest, inhibited DNA damage repair, and increased apoptosis. However, when ganetespib was combined with chemotherapy (paclitaxel and carboplatin) and radiation, not all cell lines were sensitized by ganetespib. Instead, some cell lines, for example A549, were protected by ganetespib from the toxicity of chemotherapy. This finding was also validated in the *in vivo* experiment. The animal treatments were carried out in a setting that mimicked the real clinic. Animals were treated by paclitaxel and carboplatin doublet chemotherapy once a week, in combination with daily 2 Gy fractionated radiation for 5 days, with or without ganetespib. In the H460 xenograft, chemoradiation had better tumor control efficacy compared with each treatment given alone, and the combination with ganetespib further increased the treatment outcome. However, in the A549 xenograft, ganetespib seemed to protect tumors from chemoradiation. Although

ganetespib itself demonstrated some anti-tumor effects, the combination of ganetespib with chemoradiation was even worse than chemoradiation alone. My findings indicate ganetespib may sabotage chemotherapy in some cell lines and tumors. The response to ganetespib may depend on genetic background of a tumor. Although the exact mechanism underlying the different effects of ganetespib is still unknown, this study may partially explain the negative results of the randomized phase III clinical trial of ganetespib combined with docetaxel for second line treatment of NSCLC. Therefore, in the drug screening studies, it is most ideal to screen in the condition that will be used clinically. For example, the standard treatment for unresectable stage III lung cancer is chemoradiation, *in vitro* drug screening for sensitizers or additional combinations carried out in the condition of chemoradiation is most likely to identify drugs that may potentially enhance the current standard of care.

Second, the selective radiosensitization effect of trametinib in KRAS-LKB1 mutant NSCLC highlights the complexity of cancer cell signaling and the need to select patients based on biomarkers for precise cancer therapy. My results also suggest a tumor suppressor like LKB1 can be activated by treatment combinations to induce resistance to therapies. In KRAS-mutant NSCLC, the LKB1 mutation conferred resistance to radiation and trametinib using separately, however the KRAS-LKB1 subtype is very sensitive to the combination. Mechanistic study identified that trametinib can decrease MDM2 phosphorylation at Ser-166 therefore stabilize p53 after radiation and therefore activate a strong downstream p21 signaling to induce senescence. However, in the LKB1 wildtype cell, radiation and trametinib synergistically activate the AMPK pathway through LKB1. Activation of AMPK resulted in the induction of autophagy and rescued cells from undergoing senescence. The inhibition of autophagy by AMPK knockdown or

pharmaceutical inhibitor in LKB1 wildtypes potentiated the radiosensitization efficacy of trametinib. This finding was also validated in the syngeneic mouse models using LKR13 and LKR13-Lkb1 knockout cells. The efficacy of trametinib was tested *in vivo* with chemoradiation, which is similar to the real clinical setting for treating lung cancer. As expected, although trametinib or chemoradiation was not effective when using separately, the combination significantly inhibited the tumor growth and completely eradicated tumor in about 60% of the animals. Trametinib also inhibited metastasis of LKR13-Lkb1-KO cells. This effect was further enhanced by chemoradiation. The LKR13 cells were relatively sensitive to chemoradiation and trametinib, but the combination of the two did not confer additional benefit. Adding autophagy inhibitor HCQ significantly increased the treatment efficacy of the combination of chemoradiation and trametinib. These results reflects the complexity of cancer cell signaling. Trametinib can simultaneously regulate both the p53 and LKB1 downstream pathways. When combined with radiation, trametinib activates the tumor suppressor LKB1 to induce resistance to therapy. This study suggests the KRAS-LKB1 mutant NSCLC is potentially the subtype that will benefit from the combination treatment of trametinib and chemoradiation. It also shows that the inhibition of autophagy could be a strategy to overcome therapy resistance.

Certain limitations exist in my study. All the animal models were xenograft models of human NSCLC cell lines subcutaneously implanted in nude mice or syngeneic mouse tumor models. Although these models facilitate radiation treatment and tumor volume measurement, they do not reflect the real microenvironment in the lung. As the importance of immune system in cancer treatment is being increasingly recognized, investigation of the interaction between the combination treatments and immune

response is necessary and important. Future validation study using orthotopic lung tumor models is needed. Additionally, the p53 and Lkb1 CRISPR knockout cells generated form the Kras-mutant LKR13 cell provided a controlled genetic background to investigate the function of the two tumor suppressors in response to trametinib and radiation, however, genetic modifications in the established cancer cell line may not account for the functions of p53 and Lkb1 in the tumor initiation and evolution process. Using several KP and KL cell lines derived from genetically modified mouse model to validate the findings is needed in the future.

In sum, my study demonstrated that ganetespib and trametinib can both sensitize certain NSCLC cell lines to radiation and chemoradiation. Meanwhile, the sensitization efficacy is dependent on the genetic background of the cells. Biomarker selection for potential responding patients is needed in future clinical trials. These results also emphasize the importance of preclinical testing of novel drugs in platform in the context of clinically-relevant therapy combinations.

Chapter 5: Future Directions: Combining Immunotherapy and Radiotherapy for Cancer Treatment

Since the approval of anti-CTLA4 therapy (ipilimumab) for late-stage melanoma in 2011, the development of anticancer immunotherapy agents has thrived. The success of many immune-checkpoint inhibitors has drastically changed the landscape of cancer treatment. For some types of cancer, monotherapy for targeting immune checkpoint pathways has proven more effective than traditional therapies, and combining immunotherapy with current treatment strategies may yield even better outcomes. Numerous preclinical studies have suggested that combining immunotherapy with radiotherapy could be a promising strategy for synergistic enhancement of treatment efficacy. Radiation delivered to the tumor site affects both tumor cells and surrounding stromal cells. Radiation-induced cancer cell damage exposes tumor-specific antigens that make them visible to immune surveillance and promotes the priming and activation

of cytotoxic T cells. Radiation-induced modulation of the tumor microenvironment may also facilitate the recruitment and infiltration of immune cells. This unique relationship is the rationale for combining radiation with immune checkpoint blockade. Enhanced tumor recognition and immune cell targeting with checkpoint blockade may unleash the immune system to eliminate the cancer cells. However, challenges remain to be addressed to maximize the efficacy of this promising combination. Here we summarize the mechanisms of radiation and immune system interaction, and we discuss current challenges in radiation and immune checkpoint blockade therapy and possible future approaches to boost this combination.

5.1 Introduction

The success of immunotherapy in treating some form of cancer has greatly encouraged researchers and clinicians to combine it with other conventional therapies to improve effectiveness still further. One such therapy, radiation, is commonly used to treat many types of cancer, and its combination with immunotherapy is considered promising. This combination is expected to have synergistic effects stemming from both local and systemic tumor control due to the unique and intriguing interactions between radiation and the immune system (124-126). Radiation's local therapeutic effects result from direct damage to cancer cells causing cell death and from triggering activation of $CD8^+$ T cells (127). On the other hand, the systemic immune response can also be triggered by radiation-induced microenvironmental changes to tumor cells as well as the surrounding stromal cells (126). Thus the combination of radiation and immunotherapy could be more potent than either treatment alone, as has been shown in preclinical models (128-130). The benefits of combining radiation and immunotherapy have been reported in several case reports for different cancer types, including head and neck squamous cell carcinoma (131), metastatic pancreatic cancer (132), metastatic melanoma (133), lung cancer (134), and brain metastases (135). Currently, there are numerous ongoing clinical trials testing the combination of immunotherapy and radiation (136-138). Recent results of the phase III randomized trial (PACIFIC) testing the role of the PDL1 antibody durvalumab versus placebo as consolidation therapy after chemoradiation for stage III non-small cell lung cancer (NSCLC) demonstrated substantial improvement in progression-free survival (PFS) with durvalumab (16.8 months vs. 5.6 months with placebo), with similar types and severity of side effects (139). Although these results

were impressive and will likely change the standard of care in stage III NSCLC, challenges remain for the future development of this combination therapy.

5.2 Mechanistic Rationale for Combining Radiation with Immunotherapy

5.2.1 Radiation increases antigen visibility

During the development of cancer, the relationship between the tumor and the host immune system evolves from one in which the tumor cells are recognized and destroyed by the immune system (immune elimination) to immune equilibrium, where tumor cells and immune system coexist, and finally to immune escape. The immune escape stage is characterized by upregulated inhibitory ligands and cytokines, reduced MHC class I expression, and increased numbers of myeloid-derived suppressor cells (140). This overall immunosuppressive environment causes poor antigen presentation and masks the tumor from immune surveillance and elimination. However, radiation may “unmask” the tumor, making it visible to both the innate and adaptive immune systems (126). The first step in this process is the activation of downstream immune responses and priming of T cells, which requires that antigen-presenting cells engulf the tumor cells and present their antigens to naïve T cells through phagocytosis. The presence of the calcium-binding protein calreticulin is a key signal to promote phagocytosis (141). In one study, targeting HER2-positive tumors with a multivalent bi-specific nanobioconjugate engager conjugated with calreticulin protein increased phagocytosis of tumor cells by macrophages and enhanced the priming of T cells (142). Radiation seems to promote the translocation of calreticulin from the endoplasmic reticulum to the plasma membrane (143). Meanwhile, the protein that triggers the anti-phagocytosis signal CD47 may be downregulated upon radiation exposure (144). Another key factor modulating activation of an immune response, high mobility group box 1 (HMGB1), is released from tumor cells upon exposure to x-ray or carbon-ion radiation (145). In short, radiation acts to enhance the clearance of damaged tumor cells by the antigen-presenting cells, thereby

promoting the priming of T cells. Second, downregulation of MHC-I expression on tumor cells, typical of several types of cancer, causes poor recognition of those cancer cells by the cytotoxic T cells (146). Radiation can upregulate the expression of MHC-I on the tumor surface to enable better presentation of tumor-specific peptides, enhancing the visibility of the tumor to cytotoxic T cells (147). By inducing a systemic increase in antigen recognition, radiation may also induce the T cell-mediated inhibition of untreated distant tumors (known as the abscopal effect) (148). The ability to increase tumor antigen presentation also makes radiation a promising modality to be combined with Chimeric Antigen Receptor (CAR) T-cell therapies. CAR T-cells are considered as a "living drug", since the therapy utilizes T cells isolated from patients and genetically engineers the T cells to express CAR that recognize tumor-specific antigens. Once infused back to patients, CAR T-cells are able to recognize tumor cells and induce cell death. Two CAR T-cell therapies have been approved for treating acute lymphoblastic leukemia and advanced lymphoma. However, using CAR T-cell therapies for solid tumors could be challenging due to difficulties in target selection (149). Radiation could increase and MHC-I expression and tumor-specific antigens to make the tumors a more feasible target of CAR T-cells (149). Third, radiation-induced DNA damage may generate neoantigen and trigger the immune surveillance. It is recently reported that DNA mismatch repair-deficient cancer cells grew poorly in immunocompetent mice but not in immunocompromised mice. The accumulated DNA mutations in cells with DNA repair deficiency increased the burden of neoantigens and triggered the immune response (150). It is plausible that the DNA damage induced during the course of radiotherapy may also enhance the mutational load and provide neoantigen for immune recognition, particularly in combination with DNA repair inhibitors.

However, the combination of radiotherapy with immunotherapy could be a double-edged sword. Since immune checkpoint blockade changes the equilibrium between immunity and tolerance, a higher rate of immune reaction with normal tissues accompanies with the increased likelihood of tumor recognition. Clinically, patients treated with immune checkpoint inhibitors could have immune-related adverse events (irAEs), such as fatigue, rash, skin disorders, colitis, and GI events (151, 152). When the tumor is treated by radiation, not only tumor-specific antigens but also non-tumor-specific antigens could be released into the tumor microenvironment. Some of the non-tumor-specific antigens might prime auto-reactive T cells which will attack and damage normal tissues if not properly negatively selected (153). Recent retrospective studies indicate the adverse events were increased when immunotherapies were combined with EGFR-TKI for NSCLC (154) or with radiation for brain metastases (155). These findings warrant preclinical studies to investigate the biological mechanisms underlying the increased toxicity and potential methods to lower such risks. Future prospective clinical studies are needed to improve our understanding of the benefits and risks associated with such combinations.

5.2.2 Radiation activates the cGAS-STING pathway to trigger immune responses

Radiation not only kills tumor cells directly but also seems to activate innate and adaptive immune responses through the Stimulator of Interferon Genes (STING) - mediated DNA-sensing pathway. STING is essential to protect hosts from DNA pathogens (156, 157). When the presence of cytoplasmic DNA is detected, the product of cyclic GMP-AMP synthase (cGAS), cyclic GMP-AMP (cGAMP), activates STING to

upregulate transcription of a type I interferon gene through a STING-TBK-IRF3-NFκB signaling pathway (158-161). The STING pathway plays a critical role in anti-cancer immunity, as this pathway has been reported to be frequently lost in cancers including colorectal carcinoma and melanoma (162, 163). The STING pathway is essential for radiation-induced, type I interferon-dependent antitumor immunity (164). Silencing of cGAS in bone marrow-derived dendritic cells was shown to impair their T-cell priming function when they were co-cultured with irradiated cells (164). Because of the growing evidence of STING's critical role in anti-tumor immunity, STING agonists could be promising cancer therapeutics which have been investigated in preclinical and clinical studies. It has been demonstrated that direct STING activation by intra-tumoral administration of STING agonist resulted in both local and systemic anti-tumor immune response (165). The combination of cyclic dinucleotides, a STING activator, with image-guided radiotherapy synergistically controlled both local and distant pancreatic cancer in a murine model (166). Currently, a STING agonist, MIW815 (ADU-S100), is under investigation in a phase I clinical trial (NCT02675439) to evaluate its safety and efficacy in patients with advanced solid tumors or lymphomas. However, much of the biologic mechanism of STING is still unknown. Despite numerous studies showing the immune stimulation function of STING, the role of STING pathway in anti-tumor immunity could be quite intriguing. It has been reported that STING deficiency protects mice from 7,12-dimethylbenz(a)anthracene induced skin cancer by decreasing inflammatory cytokine release (167). The STING pathway activation may also enhance indoleamine 2,3 dioxygenase activity in the tumor microenvironment and induces immune tolerance in the lewis lung carcinoma model (168). A recent study suggested that STING activation after radiation could drive immunosuppression. Radiation-induced STING and type I interferon activation recruits myeloid-derived suppressor cells to the irradiated tumor

through the CCR2 pathway, causing immunosuppression and radioresistance (169). In addition, the mechanism of how DNA released from damaged cancer cells is transferred to antigen presenting cells to activate the STING pathway is still not clearly understood (170). More studies into the biological mechanisms and the therapeutic potential of the STING pathway are still needed.

5.2.3 Radiation modifies tumor stromal microenvironments

A tumor is not an isolated island of tumor cells, but a complex organ supported by stromal cells and blood vessels (171). Stromal cells and their secreted signals (cytokines, chemokines, and growth factors) constitute the major portion of the tumor microenvironment. Several cytokines can be induced by radiation (172), one of the most critical of which is transforming growth factor β (TGF-β). TGF-β signaling is upregulated momentarily after radiation (173) and triggers an immune-suppressive microenvironment by reducing the cytotoxicity of $CD8^+$ T cells, suppressing $CD4^+$ T-cell differentiation, promoting regulatory T cell (Treg) transformation, and inhibiting natural killer (NK) cell proliferation (174-176). TGF-β is also involved in the activation of vascular endothelial growth factor (VEGF) transcription (177). Given these immunosuppressive actions of TGF-β, attempts have been made to inhibit TGF-β signaling after radiation. In one preclinical study, radiation combined with TGF-β neutralization increased T cell priming and decreased tumor growth and metastasis (178), and the addition of anti-PD1 therapy to this combination further extended the survival of the experimental mice (178).

5.3 Challenges in Combining Radiation and Immunotherapy

5.3.1 Optimizing the timing of radiotherapy and immunotherapy

In any combination treatment that involves several treatment modalities, the timing of each component could be critical to the outcome. Since different types of immunotherapy target different pathways or different immune cells, the strategy of treatment combinations should be carefully designed to produce the greatest synergistic effects (**Figure 19**). To date, several preclinical and clinical studies have been carried out to interrogate this question. So far, the results appear to suggest that the optimal timing is tumor type and immunotherapy-specific. In a mouse study testing combinations of hypofractionated radiation therapy (20 Gy) and immunotherapy drugs, anti-CTLA4 was found to work most effectively when given before the radiation, but anti-OX40 was more effective when given 1 day after the radiation (179). A study of patients with melanoma brain metastases showed that concurrent immunotherapy with anti-PDL1 and anti-CTLA4 given within 4 weeks of stereotactic radiosurgery led to improved response of brain lesions relative to treatments that were separated by more than 4 weeks (180). A preclinical mouse study showed that resistance to fractionated radiotherapy could be overcome by PDL1 blockade, but PDL1 inhibition was effective only when given either concomitantly with or at the end of radiation, not 1 week following radiation (129). The secondary analysis of the KEYNOTE-001 trial (NCT01295827) showed the NSCLC patients who received radiotherapy before pembrolizumab (anti-PD1) had better overall survival and progression-free survival compared with the patients who did not receive radiotherapy (181), suggesting radiation may enhance the efficacy of immunotherapy. Interestingly, analysis of the PACIFIC trial (139) examining the timing when durvalumab was started relative to the completion of chemoradiotherapy suggests that starting

durvalumab within 14 days after completing chemoradiotherapy appeared to have greater PFS efficacy than if durvalumab were started after 14 days. A recent retrospective review of 758 patients who received immunotherapy (anti-CTLA4 and/or anti-PD1/anti-PDL1) and radiotherapy suggested that overall survival was better for patients who received concurrent immunotherapy and radiotherapy (182). Among the patients who received concurrent therapy, survival was longer when induction immunotherapy was begun more than 30 days before radiation compared with immunotherapy begun within 30 days before radiation (median overall survival times 20 months vs. 11 months) (182). Collectively, this preclinical and clinical evidence strongly suggests that the scheduling of radiotherapy and immunotherapy must be considered carefully, ideally in the context of clinical trials. One phase II trial currently ongoing at MD Anderson Cancer Center (NCT02525757) that considers the timing of immunotherapy is evaluating the safety and efficacy of atezolizumab, a monoclonal antibody targeting PDL1, in combination with standard chemoradiation (carboplatin and paclitaxel plus conventional 2-Gy fractionated radiation) for unresectable locally advanced NSCLC. For the first treatment group, atezolizumab is given 3-4 weeks after completion of chemoradiation for up to 1 year. If the toxicity of this sequential delivery can be tolerated, the second treatment group will be given atezolizumab concurrently with chemoradiation for 6-7 weeks as well as afterwards for up to 1 year. Although this is not a randomized comparison of the two regimens, insights into how the different schedules affect the safety and efficacy of the combined treatment could be useful for future trial design.

5.3.2 Optimizing the dose of radiotherapy: conventional fractionation or hypofractionation

The radiation dose and fractionation schedule are also important factors to consider when radiation is combined with immunotherapy. The conventional fractionation scheme, that is, 1.8 to 2-Gy fraction given once a day, takes advantage of tumors' vulnerability in terms of DNA repair and cell cycle regulation. However, whether this conventional fractionation schedule, or one that utilizes a hypofractionated schedule (larger doses per day over a shorter course of time to a lower or same total dose) produces the best synergy with immunotherapy remains in question (Figure 20). Several preclinical studies have compared single high-dose radiation with fractionated radiation for their ability to induce an immune response. In a B16-OVA melanoma model, both single-dose radiation (15 Gy) and fractionated radiation (five fractions of 3 Gy each) delivered to tumors increased the generation of antigen-specific T cells. However, the 15-Gy single-dose regimen generated more tumor-infiltrating T cells than did the fractionated regimen (183). Moreover, the secretion of interferon-γ by cells in tumor-draining lymph nodes was higher in the mice given a single dose than in the mice given fractionated radiation (183). A later report of a B16 mouse model from another group also showed that the immune response triggered by ablative radiation doses was abrogated by conventional fractionation (127). In another study of a Lewis lung carcinoma (LLC) murine lung cancer model, 5 fractions of 10 Gy each induced a more robust abscopal effect than 12 fractions of 2 Gy each (184). However, evidence also exists to show hypofractionation is not favorable when combined with immunotherapy. In preclinical breast cancer models, an abscopal effect was induced only by fractionated radiation, not single-dose radiation, when given in combination with anti-CTLA4 (185). A preclinical study of human prostate cancer cells showed that exposure to multifraction radiation (ten 1-Gy fractions) induced the release of damage-associated molecular pattern molecules more robustly than did single-dose treatment (one 10-Gy fraction)

(186). In a recent study, extreme high-dose radiation (20-30 Gy in 1 fraction) was shown to sabotage tumor immunogenicity by inducing DNA exonuclease Trex1 to block cGAS-STING pathway activation (187, 188). In this study, anti-CTLA4 therapy was not able to synergize with high dose radiation to induce an abscopal effect in the TSA mouse mammary carcinoma model. The authors found that the expression of Trex1, a major 3' DNA exonuclease, was significantly upregulated when cells received more than 10 Gy of radiation. As a result, cytosolic DNA was significantly curtailed in cancer cells that received high dose radiation compared to cells that received low dose radiation, and activation of the cGAS-STING pathway to produce interferon- β was greatly attenuated. Knockdown of Trex1 enabled 20 Gy of radiation to induce the abscopal effect with anti-CTLA4 (187). Taken together, radiation dose and scheduling appear to be important factors when combined with immunotherapy, and could further be complicated by the types of immunotherapy used. Additional preclinical studies and clinical trials are needed to unravel the optimal radiation dose and scheduling that could best synergize with immunotherapies.

5.3.3 Minimizing the direct effects of radiation on T cells

The radiosensitivity of T lymphocytes makes them vulnerable targets during radiation therapy. Tumor-infiltrating T cells are inevitably irradiated, especially during prolonged courses of radiation, and the conventional 2-Gy once-daily schedule could be sufficient to inactivate T cells (189). These findings raised concern that conventional fractionation may inhibit T cells inside the tumor. In addition to directly irradiating tumor-infiltrating T cells, radiation may also negatively impact T cells in the peripheral blood that transit through the irradiated field (Figure 21). It has been reported that radiation induces

lymphopenia in patients, and that severe lymphopenia was associated with poor prognosis in non-small cell lung cancer and nasopharyngeal cancer (190, 191). We recently found that patients with esophageal cancer had high incidence of grade 4 lymphopenia during chemoradiation therapy that was not apparent when chemotherapy was given alone (192). Because most immunotherapies depend on functioning T cells, lymphopenia is likely to undermine immunotherapy efficacy. The risk of developing lymphopenia could also be associated with the radiation modality. In the study mentioned above (192) and a propensity matched follow-up study (193), we found proton radiation was significantly associated with reduced grade 4 lymphopenia risk for esophageal cancer patients treated by neoadjuvant or definitive chemoradiotherapy. The dosimetric advantage of proton therapy, which spares surrounding normal tissue from radiation, may be the main reason for the reduced risk of lymphopenia. Whether immunotherapy better synergizes with proton radiation compared with photon therapy needs further investigation. Irradiation of lymph nodes is also an issue to consider, because even though nodal radiation is known to enhance local control in node-positive disease, nodal irradiation presumably would affect immune-specific T cells in the draining lymph nodes. In a preclinical study examing the effects of prophylactic nodal irradiation, mouse models of both colon cancer and melanoma were used to compare tumor growth and tumor-infiltrating lymphocytes after irradiation of just the tumor or the tumor and the draining lymph nodes (194). The inclusion of the draining lymph nodes in the radiation field did not affect tumor control, but it did reduce the proportion and absolute numbers of tumor-infiltrating $CD8^+$ T cells. However, irradiation of the draining lymph nodes increased the levels of T-cell chemoattractants and antigen-specific $CD8^+$ cells in the tumor microenvironment (194), pointing to the complexity of T cell dynamics after nodal irradiation. Whether nodal irradiation synergizes or sabotages checkpoint

inhibitors such as anti-CTLA4 or anti-PDL1 remains to be seen, and needs further preclinical and clinical investigation.

5.3.4 Identifying biomarkers to predict responders to combination therapy

Despite remarkable successes in the past decade, the effectiveness of immunotherapy varies across patients and across cancer types (195). Currently, increasing the response rates to immunotherapy and identifying biomarkers with which to predict which patients are most likely to respond to immunotherapy represent unmet needs for the full implementation of precision immunotherapy (Figure 22). To date, biomarkers tested for their ability to predict immunotherapy responses include PDL1 expression (196-199), mutational burden and neoantigens (200-204), DNA repair deficiencies (205, 206), tumor-infiltrating lymphocytes (207, 208), patient HLA class I genotype (209), and serum markers (210-213). Recent studies have suggested that the response to immunotherapy could be predicted by the gut microbiome in mouse models (214) and melanoma patients (215). The latter study showed the melanoma patients with more diverse and abundant Ruminococcaceae bacteria responded better to anti-PD-1 therapy. More importantly, the fecal transplant from responding patients to germ-free mice enhanced both systemic and anti-tumor immunity. These findings not only reveal the utility of the gut microbiome as a predictive marker for immunotherapy effectiveness, but also support the notion that immunotherapy response could be altered by the adoptive transfer of the gut microbiome to non-responding patients as a novel therapeutic approach.

Actually, the greatest challenge in predicting response to combination therapy could be that the combination itself may affect the signaling network of the tumor so as to alter its behavior and response to treatment. For example, in preclinical models, the HSP90 inhibitor ganetespib was able to radiosensitize a panel of NSCLC cell lines with diverse genetic backgrounds. However, when tested with chemoradiation, ganetespib could sensitize some cell lines, but not others, both *in vitro* and *in vivo* (52). Because predicting response to immunotherapy is itself a challenge, identifying biomarkers to predict outcomes after combined immunotherapy and radiation could be far more challenging. A recent study in which mass cytometry was used to profile immune-cell infiltrates after treatment with either of two checkpoint inhibitors (anti-CTLA4 and anti-PD1) showed that the antitumor effects of each were driven by distinct mechanisms of action (216). The responses to immunotherapy also vary across cancer types. A recent genomic analysis of 100,000 human cancers showed the diverse mutation burden across different cancers and the cancer types that had high mutation burdens generally had better responses to immunotherapies, such as melanoma, NSCLC, bladder cancer, and renal cell carcinoma (217). Because of the complexity of responses for different types of checkpoint blockades and cancers, identifying a “universal marker” that predicts response to all types of checkpoint blockade therapies in all cancer types may not be possible. In addition, several novel checkpoints are emerging in recent years (218), whether radiation synergizes with them still needs to be investigated. Moreover, responses to immunotherapy may emerge later than responses to conventional chemotherapy or other targeted therapies, and thus the criteria and standards for evaluating “response” is still a matter of debate (195).

Figure 19. Optimization of treatment timing: using immunotherapy concurrently, sequentially, or as neoadjuvant therapy with radiotherapy.

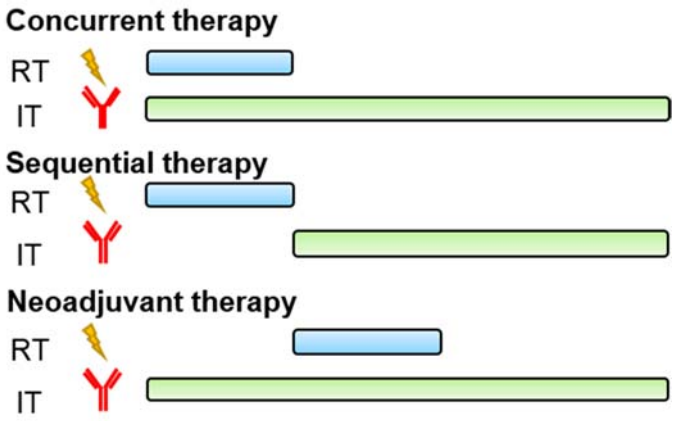


Figure 20. Optimization of radiation dosing: conventional fractionation or hypofractionation.

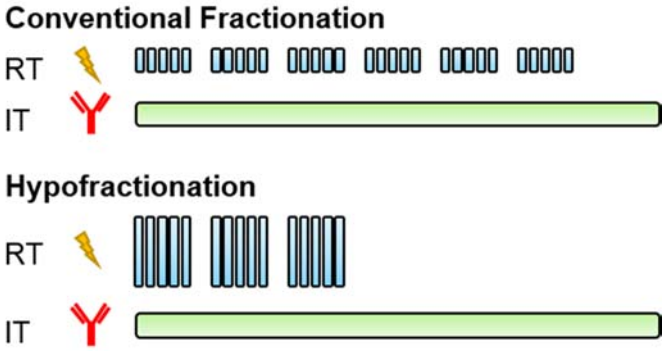


Figure 21. Reduction of the radiation-induced toxicity of circulating and tumor-infiltrated lymphocytes.

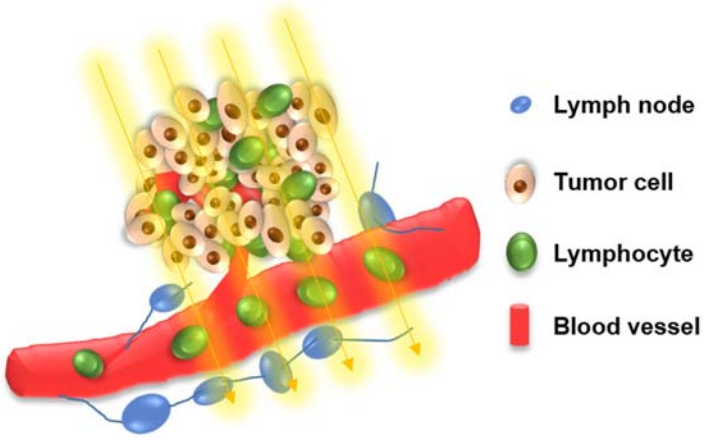
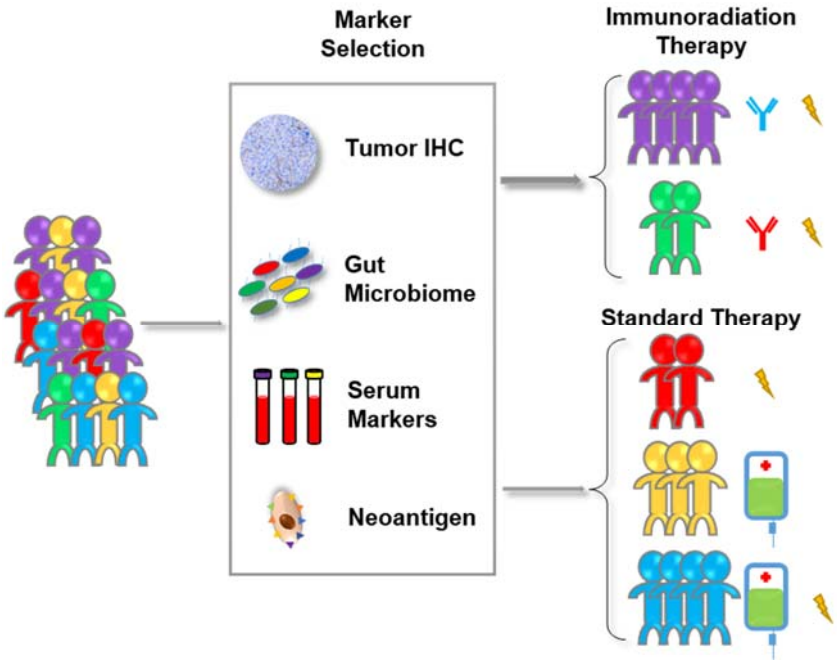


Figure 22. Selection of immunoradiation therapy or standard therapy for patients based on predictive biomarkers.



5.4 In the Future: Could Immunotherapy be a Radiation Sensitizer?

To date, discussions of synergy between radiation and immunotherapy have focused mostly on how radiation could enhance the therapeutic effects of immunotherapy, as described previously in this current review. However, whether immunotherapy itself could be a radiation sensitizer has not been widely investigated. Radiosensitization agents increase a tumor's sensitivity to radiation, with the promise of enhancing cytotoxicity to the tumor without the need of higher radiation doses. Chemotherapy, monoclonal antibodies, and targeted agents all have radiosensitization effects in several types of tumor (52-57). Indeed, the relationship between radiation and immunotherapy may be more profound and complex than had previously been thought. One might assume that immunotherapy could sensitize tumor cells to radiation on the basis of current knowledge as follows. First, several regulators of both radiosensitivity and immune checkpoints have been identified, among them PARP inhibitors (58), which may act by upregulating PDL1 expression and inducing immunosuppression (219). Another well-known radiation response regulator, p53, had also been shown to modulate PDL1 expression (220). Second, immune checkpoint blockade may influence the tumor microenvironment by regulating cytokine secretion (221, 222) and by remodeling tumor vasculature (223). Immunotherapy could plausibly affect tumor radiation response through mechanisms that are independent of their effects on immune cells. Given the scarcity of evidence that immunotherapy may have direct or indirect radiosensitizing properties, preclinical and clinical studies will be helpful to ascertain this possibility.

5.5 Conclusion

In summary, radiation seems to synergize with immunotherapy via several mechanisms, such as increasing the visibility of tumor antigens, activating the cGAS-STING pathway, and modulating the tumor microenvironment. Although the combination of radiation and immunotherapy has proven effective in preclinical studies and shows promise in clinical trials, challenges still exist for the future application of this combination therapy. The optimization of radiation dose and timing and the identification of potential biomarkers may further enhance the effectiveness of this unique combination. In the meantime, the concept that immunotherapy may act as a radiation sensitizer to improve tumor local control could be another fruitful avenue of investigation.

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