Radiation-Enhanced Lung Cancer Progression in a Transgenic Mouse Model of Lung Cancer Is Predictive of Outcomes in Human Lung and Breast Cancer

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Abstract

Purpose: Carcinogenesis is an adaptive process between nascent tumor cells and their microenvironment, including the modification of inflammatory responses from antitumorigenic to protumorigenic. Radiation exposure can stimulate inflammatory responses that inhibit or promote carcinogenesis. The purpose of this study is to determine the impact of radiation exposure on lung cancer progression in vivo and assess the relevance of this knowledge to human carcinogenesis.

Experimental Design: K-rasLA1 mice were irradiated with various doses and dose regimens and then monitored until death. Microarray analyses were performed using Illumina BeadChips on whole lung tissue 70 days after irradiation with a fractionated or acute dose of radiation and compared with age-matched unirradiated controls. Unique group classifiers were derived by comparative genomic analysis of three experimental cohorts. Survival analyses were performed using principal component analysis and k-means clustering on three lung adenocarcinoma, three breast adenocarcinoma, and two lung squamous carcinoma annotated microarray datasets.

Results: Radiation exposure accelerates lung cancer progression in the K-rasLA1 lung cancer mouse model with dose fractionation being more permissive for cancer progression. A nonrandom inflammatory signature associated with this progression was elicited from whole lung tissue containing only benign lesions and predicts human lung and breast cancer patient survival across multiple datasets. Immunohistochemical analyses suggest that tumor cells drive predictive signature.

Conclusions: These results demonstrate that radiation exposure can cooperate with benign lesions in a transgenic model of cancer by affecting inflammatory pathways, and that clinically relevant similarities exist between human lung and breast carcinogenesis. Clin Cancer Res; 20(6); 1610–22. ©2014 AACR.

Introduction

Carcinogenesis is a complex multistep process driven by the convergence of both intrinsic and extrinsic factors. DNA mutations that result in cellular dysregulation through the activation of an oncogene or the silencing of a tumor suppressor gene are classic examples of intrinsic factors. Extrinsic factors are those provided by physical and biochemical interactions between the emerging tumor and its microenvironment (1–4). Reciprocal interactions between these factors are dynamic throughout carcinogenesis and readily influence overall tumor responses (2, 4, 5). For processes, such as inflammation, this may result in pleiotropic or conflicting responses dependent on the initiated tissue type, mutational landscape of the tumor, and/or signal duration and intensity (2, 5, 6). The overall impact of modifying these factors, especially those regulating inflammation, may, therefore, fluctuate contextually throughout the carcinogenic process.

Radiation exposure is a known carcinogen in which effects may likewise be contradictory and contextual. The classical method in which radiation is thought to act as a carcinogen is intrinsic through the induction of DNA damage leading to an increased mutational load, chromosomal aberrations, and/or genomic instability (1, 3, 6). These effects are generally observed following the exposure to
Translational Relevance

Radiation exposure is often used therapeutically as it readily induces DNA damage. However, radiation is a known carcinogen that is increasingly being used as a diagnostic tool and, therefore, may pose a potential risk to human health. In this study, we sought to determine how radiation exposure affects cancer promotion and progression in vivo through the use of a mouse model of lung cancer and whether any gender or genetic background influences exist. Our results demonstrate that radiation exposure enhances lung cancer progression in the K-rasLA1 mouse model of lung cancer. A nonrandom signature that is predictive of both human lung and breast cancer survival was elicited after irradiation from murine lung tissue containing only benign lesions. This study provides evidence that the dysregulation of networks important for cancer progression and patient survival may be detectable in histologically identical tissues and that radiation exposure may cooperate with these networks for malignant transformation.

lower radiation doses as the exposure to higher doses is capable of inducing cell death (1, 3). Evidence is mounting, however, that radiation exposure can also modify extrinsic factors by disturbing cells in the microenvironment. These cells, in turn, affect the adjacent tumor cells in a cell nonautonomous fashion (1, 3, 7–9). Empirical evidence for this extrinsic role of radiation exposure on carcinogenesis has been largely derived from studies that rely on the use of normal and/or cancer cells in cell culture or transplantation assays (7–10). It is not clearly understood whether initiated, nontransformed cells equally respond to these radiation-induced cues or whether the transplantation procedure introduces extraneous damage that cooperates with radiation exposure.

Ionizing radiation is composed of both electromagnetic and particulate radiation types, with the risk of exposure being higher for electromagnetic radiation types. The majority of radiation studies have examined the carcinogenic effect of electromagnetic radiation exposure and, as such, these effects are used as the baseline for determining the biologic effectiveness of other radiation types (1). The therapeutic application of high-energy particle radiation and the mounting interest for deep space travel, however, is increasing the population exposed to high-energy particulate radiation. The energy spectrum is lower than other charged particle types and that of high-energy neutron particles in space. Studies examining the effect of fractionation on high-energy charged particles or directly comparing acute and fractionated doses on promotion and progression in vivo are limited. Therefore, how dose fractionation affects these stages of the carcinogenic process is not fully understood.

In this study, we examined the effect of radiation exposure on the later stages of the carcinogenic process using a lung cancer susceptible mouse model, K-rasLA1, in which lesions are spontaneously activated (15). Our results provide evidence that both electromagnetic and particulate radiation exposure is capable of accelerating lung cancer progression and that dose fractionation creates a more permissive environment for this progression. Comparative genomic analysis between whole lungs from unirradiated K-rasLA1 animals and those exposed to a fractionated or acute dose of high-energy particulate radiation revealed an expression signature that is capable of segregating K-rasLA1 animals irradiated with a fractionated dose from all others. This murine-derived “fractionated” gene classifier, which is driven by inflammatory networks, demonstrates relevance to human carcinogenesis as it retains the capacity to predict overall survival for patients with human lung and breast cancer. Therefore, these results strongly support the concept that radiation exposure can enhance cancer progression through the disruption of inflammatory responses and identify an underlying biology related to inflammation with clinical relevance for both human lung and breast cancer.

Materials and Methods

Study design

Irradiation studies were initiated to evaluate impact of radiation exposure on later stages of carcinogenesis in vivo. Experiments were initiated with a tolerable dose to wild-type mice, 1.0 Gy total dose, as there were no data on how radiation exposure would affect the carcinogenic process. Ensuing experiments were designed to assess the impact of radiation exposure at lower doses in addition to the impact of each dose fraction tested as an acute dose. Sample sizes for irradiation studies were established via power analysis of published survival data for K-rasLA1 mice. Cohort sizes were adjusted during the course of the study to accommodate the progression phenotype observed and were based on power analysis of background incidence of invasive adenocarcinoma in K-rasLA1 mice. Animals were randomly assigned to all experimental cohorts, irradiated as described below, and monitored until death or euthanization due to health...
Concerns. Euthanized animals were censored in corresponding survival analyses and all histopathologic analyses were blinded. For microarray studies, cohort sizes were determined on the basis of the power analysis of previous in-house expression data obtained from K-ras<sup>LA1</sup> mice. Endpoint determination was guided by literature on studies examining the resolution of lung damage and overall radiation-induced effects in mice. Extraction and processing of all samples were blinded and then randomized across microarray chips. Samples for Western blot and immunohistochemistry (IHC) analyses were additionally extracted and processed blinded, as was the quantification of immunohistochemical results.

**Mice**

Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center at Dallas (UTSW; Dallas, TX) and the Brookhaven National Laboratory (BNL; Upton, NY). B6.129S2 K-ras<sup>LA1</sup> mice were obtained from the National Cancer Institute (NCI) Mouse Models of Human Cancers Consortium (Fredrick, MD) and 129S2 K-ras<sup>LA1</sup> mice from Dr. Jonathan Kurie (the University of Texas MD Anderson Cancer Center, Houston, TX). Animals were housed and bred in ventilated microisolator cages within a specific pathogen-free (SPF) facility at UTSW. Heterozygote K-ras<sup>LA1</sup> mice were crossed with wild-type animals of the same background.

**Irradiation**

Male and female K-ras<sup>LA1</sup> mice, ages 5 to 15 weeks, were irradiated whole body with either 250-kV X-rays or 1.0 GeV/nucleon <sup>56</sup>Fe-particles and then monitored twice daily in UTSW SPF facility until death or euthanasia due to health concerns. X-ray experiments were performed at UTSW with X-RAD 320 irradiator (Precision X-ray, Inc.) at an approximate dose rate of 0.14 Gy/min. For <sup>56</sup>Fe-particle irradiation, animals were shipped to BNL, irradiated in the NASA Space Radiation Laboratory at dose rate of 0.2 Gy/min, and then returned to UTSW SPF facility following required quarantine period.

**Lung tumor histology and evaluation**

At necropsy, the lungs were extracted, inflated via intratracheal infusion with 10% neutral buffered formalin (NBF), and then, after clamping the trachea, whole tissues were immersion-fixed overnight in 10% NBF. Liver, kidneys, spleens, and any tissues displaying gross abnormalities were also extracted and immersion-fixed overnight. Tissues were processed, paraffin-embedded, and 5-μm sections were stained with hematoxylin and eosin (H&E) for blinded histopathologic evaluation. Hematologic disorders required confirmation in one or more tissues. To quantify tumor size and number, three sections were cut per animal approximately 50-μm apart. Sections were H&E-stained and images of all discernable lesions were captured with Axiosvision software v4.6.3 on Axioskop 2 plus microscope mounted with AxioCamHR color camera (Carl Zeiss Microscopy). Measurement of the surface area was performed with ImageJ software (16).

**RNA extraction**

Age-matched 129S2 K-ras<sup>LA1</sup> mice were sent to BNL and irradiated with an acute dose of 1.0 Gy <sup>56</sup>Fe-particles, 5 daily doses of 0.2 Gy <sup>56</sup>Fe-particles, or left unirradiated. Seventy days after irradiation, the lungs were extracted and flash-frozen in liquid nitrogen. Frozen tissue was homogenized and RNA extracted with the Qiagen RNeasy Plus Kit (Qiagen) per the manufacturer’s protocol.

**Microarray and survival analyses**

Mouse microarrays were performed using Illumina MouseWG-6 v2.0 Expression BeadChips (Illumina). Samples were labeled and hybridized using the Illumina TotalPrep Kit (Ambion) and then arrays were scanned using Illumina Beadstation 500 BeadArray reader and data acquisition with BeadStudio (Illumina). R 2.15.1 (http://www.R-project.org/) and tools in Bioconductor (http://www.bioconductor.org/) were used for all analyses unless otherwise stated. More detailed methodology, including flowchart and Sweave report, for microarray and survival analyses in Supplementary Methods.

**Western blot analyses**

Age-matched 129S2 K-ras<sup>LA1</sup> mice were irradiated, lungs extracted, and flash-frozen 70 days after irradiation as described above for RNA extraction. Frozen tissue was homogenized on ice in cold lysis buffer (50 mmol/L Tris-HCl, pH 7.5; 120 mmol/L NaCl; and 1 mmol/L EDTA) supplemented with PhosStop phosphatase inhibitor and Complete protease inhibitor cocktail tablets (Roche). Immediately following homogenization, samples were mixed well with Triton X-100 (1% final concentration; Sigma-Aldrich) and incubated on ice for 20 minutes. Lysates were centrifuged at 13,000 rpm for 20 minutes and supernatants transferred to new tubes. Protein concentration was determined via Bradford assay (Bio-Rad) and 15 μg were separated on 4% to 15% Criterion TGX gels (Bio-Rad). Gels were transferred onto nitrocellulose membranes (Bio-Rad) with the Trans-Blot Turbo Transfer System (Bio-Rad) and blocked with 5% milk. Blots were incubated with primary antibodies, diluted in 1× PBST, overnight at 4°C (MAPK14 Cat# 9212, phospho-p38 MAPK Cat# 9211, JNK Cat# 9165, phospho-JUN Cat# 3270, phospho-STAT3 Y705 Cat# 9145, and phospho-STAT3 S727 Cat# 9134 (Cell Signaling Technology); STAT3 Cat# 06-596 (Millipore)). After three washes with 1× PBST, blots were incubated with secondary antibody (horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG or goat anti-rabbit IgG) diluted in 5% milk for 1 hour at room temperature and then washed again with 1× PBST. Blots were exposed with SuperSignal West Femto Chemiluminescent Substrate diluted 1:5 in dH<sub>2</sub>O (Thermo Fisher Scientific). Images were acquired with GeneSnap software (Syngene) on the G:BOX Chemi system (Syngene) and quantified using GeneTools software (Syngene).
Immunohistochemistry

Age-matched 129S2 K-rasLA1 mice were irradiated and, 70 days after irradiation, lungs extracted and fixed with 10% NBF as described above for lung tumor evaluation. Tissues were processed, paraffin-embedded, and cut in to 5-μm sections. Sections were deparaffinized, rehydrated, and then antigens retrieved with citrate buffer (10 mmol/L sodium citrate, pH 6.0; 0.05% Tween 20) in pressure cooker. Endogenous peroxidase, biotin, and proteins were sequentially blocked with solutions of 3% hydrogen peroxide (Sigma-Aldrich), the Avidin/Biotin Blocking Kit (Vector Laboratories), and 10% bovine serum albumin (Vector Laboratories). Primary antibodies were diluted in 1× TBST with 5% bovine serum albumin and then sections were incubated overnight at 4°C (phospho-JUN Cat# 3270; phospho-STAT3 Y705 Cat# 9145). After three washes with 1× TBST, secondary antibody and ABC reagent were applied using the VECTASTAIN ABC Kit (Vector Laboratories) following the manufacturer’s protocol. Tissue sections were then incubated with ImmPACT DAB peroxidase substrate (Vector Laboratories), counterstained with methyl green, and then dried overnight before mounting coverslip. Images were captured with Axiovision software v4.6.3 on Axiostar plus microscope mounted with AxiocamHR color camera (Carl Zeiss Microscopy) using Plan-APOC-40× objectives. Quantification was performed on 20× fields of view using ImageJ software (16) by isolating the DAB and methyl green signals using the Colour Deconvolution plugin and then quantifying each signal independently. Entire fields were quantified for non-involved regions, whereas only cells within tumor margins were quantified for hyperplasias and adenomas.

Statistical analysis

Statistical analysis of histopathology was performed using the two-tailed Fisher exact (95% confidence interval, CI) or χ² test when appropriate. For age adjustment, all animals older than 500 days were censored before statistical analysis. Gender and background effects were evaluated for individual groups by univariate Cox regression analysis and between groups by either logistic regression or multivariate Cox regression analysis. Differences in survival were determined by the log-rank test. Analysis of Western blots was through one-way ANOVA with Tukey correction. Tumor burden and IHC analysis was by two-way ANOVA with Tukey correction.

Results

Radiation exposure enhances lung cancer progression in the K-rasLA1 mouse model

To determine the impact of radiation exposure on animals predisposed to the development of lung cancer, 5- to 15-week-old K-rasLA1 mice were whole-body irradiated with varying doses of X-rays or high-energy 56Fe-particles. In this mouse model, pulmonary lesions are initiated through the spontaneous activation of a latent mutant K-ras G12D allele and predominantly result in the development of benign lung adenomas (15). Only 50% of K-rasLA1 mice will develop adenomas with atypia and, even fewer K-rasLA1 mice, 18%, will have lesions that will fully progress to invasive lung adenocarcinoma (Fig. 1A–F). These adenocarcinomas are characterized by tumor cells with high nuclear-to-cytoplasmic ratios, definite nuclear pleomorphism, and have an undifferentiated appearance (Fig. 1E and Supplementary Fig. S1). Various adenocarcinomas also demonstrate metastatic potential (Supplementary Fig. S1). There is a marginally significant impact of the genetic background on the incidence of invasive carcinoma in this model as a higher incidence was observed in K-rasLA1 mice backcrossed to a C57BL/6 background compared with those backcrossed to a 129S2 background (Supplementary Table S1). A general trend for a gender effect was also observed with females being more susceptible to this transformation (Supplementary Table S1).

Although radiation exposure at the doses used in these studies did not affect the overall incidence of adenomas or adenomas with atypia in this model, the overall incidence of invasive adenocarcinoma was substantially increased following some acute and fractionated irradiation regimens (Fig. 1F). Logistic regression analysis to assess the impact of strain and sex on this increased incidence of invasive adenocarcinoma demonstrated an influence of strain as radiation exposure differentially increased the overall incidence in each mouse strain (OR, 4.00; 95% CI, 0.95–16.92 vs. OR, 1.90; 95% CI, 1.27–2.85; Supplementary Tables S1–S3). Gender did not significantly impact this effect (Supplementary Tables S2 and S3). Irradiation of K-rasLA1 mice with either an acute or fractionated dose of 1.0 Gy 56Fe-particles resulted in a significant decrease in survival, which was also influenced by strain (Fig. 1G and Supplementary Tables S4 and S5). This is in contrast with the survival of unirradiated K-rasLA1 mice, which is not affected by either strain or sex (HR, 0.97; 95% CI, 0.53–1.75; HR, 1.4; 95% CI, 0.81–2.53, respectively). An age adjustment to account for this radiation-induced decrease in survival did not impact the effect of radiation exposure on the incidence of invasive carcinoma (Fig. 1F).

Approximately 25% of K-rasLA1 mice possess lung tumors that extend into the bronchial airways and these extensions were also more frequently observed in K-rasLA1 mice following irradiation with 56Fe-particles or a fractionated 2.0 Gy dose of X-rays (Supplementary Fig. S2A and Supplementary Tables S1 and S2). The incidence of pneumonia is highly correlated with that of bronchial extensions in K-rasLA1 mice (P < 0.0001; χ² test), but neither radiation exposure nor the increase in obstructed airways was found to affect the incidence of pneumonia (Supplementary Fig. S2B and Supplementary Tables S1 and S2). K-rasLA1 mice are also susceptible to thymic lymphoma (15) and radiation is known to affect the health of mice by inducing hematologic malignancies, including lymphoma (17). Examination of the incidence of lymphoma, leukemia, and myeloproliferative disorder revealed that the overall incidence of individual hematologic malignancies was modestly suppressed in K-rasLA1 mice following various
irradiation regimens (Supplementary Fig. S2C–S2F and Supplementary Tables S1 and S2). The combined incidence of hematologic malignancies, however, did not seem to be influenced by radiation exposure (Supplementary Fig. S2E) and, thus, may not directly contribute to the effect of radiation on survival or lung cancer progression in the K-rasLA1 mouse model. Interestingly, there was an inverse correlation found between the incidence of lymphoma and leukemia and invasive lung adenocarcinoma in these mice ($P < 0.0001; \chi^2$ test).

$^{56}$Fe-charged particle irradiation increases the incidence of hepatocellular carcinoma in the CBA/CaJ mouse strain, which has a relatively high background incidence, whereas X-ray irradiation has only a minimal effect (18). Although, K-rasLA1 mice are not susceptible to liver carcinogenesis, a similar trend was observed with the detection of small hyperplastic nodules or hepatomas in livers following exposure to $^{56}$Fe-particle radiation (Supplementary Fig. S2G).

**Irradiated K-rasLA1 lungs have distinct expression profiles dependent on dose regimen**

To examine global gene expression changes after irradiation. This was performed at the tissue level so that any influence of the microenvironment would also be included. To eliminate any strain effects, only K-rasLA1 mice backcrossed into a 129S2 background were used because unirradiated mice of this background are less susceptible for transformation (Supplementary Table S1). Of particular interest for this analysis was the differential effect of 1.0 Gy $^{56}$Fe-particle irradiation on K-rasLA1 mice. Radiation exposure resulted in a significant decrease in survival of K-rasLA1 mice irrespective of dose regimen (Fig. 1G), whereas only K-rasLA1 mice irradiated with a fractionated dose demonstrated an increased incidence of invasive lung adenocarcinoma (Fig. 1F). Therefore, the effect of radiation on K-rasLA1 survival and lung cancer progression may be in part due to distinct biologies that are intertwined and collectively influence each phenotype.

Tumor cells and their microenvironment are known to change throughout the carcinogenic process (2, 4). To determine how radiation exposure affected the carcinogenic process, with minimal confounding influences from the resultant cancer, total RNA was extracted from K-rasLA1 lungs 70 days after irradiation with an acute or fractionated dose of 1.0 Gy $^{56}$Fe-particles and compared with age-matched
This allotted sufficient time for irradiated lungs to recover from any early radiation damage while short enough that tissues/tumors from all cohorts remained histologically identical (Fig. 2). At this time point, no advanced adenomas or carcinomas were observed in either control or irradiated mice and inflammatory infiltrates, such as lymphocytes and macrophages, were negligible (Fig. 2). Thus, any molecular changes observed would be predicted to be directly associated to changes in the evolving carcinogenic process.

After background normalization and outlier exclusion, we applied a variation-based filter to eliminate any noise potentially introduced by tissue similarities and cellular heterogeneity (see Supplementary Methods and Supplementary Fig. S3). This resulted in 4,580 unique probes representing 4,311 genes available for analysis.

Although experimental groups could not be distinguished histologically, collective differences in gene expression were sufficient to segregate these groups by either hierarchical clustering (HCL) or principal component analysis (PCA) using 632 genes (Supplementary Figs. S3 and S4). This large number of genes required for this segregation further signifies the overwhelming complexity inherent to the genetic analysis of multicellular tissues that do not have overt histologic differences. Interrogation of the underlying biology encompassed by this gene signature with Ingenuity pathway analysis (IPA) revealed that these genes could be ascribed to several networks associated with cancer, organismal injury and abnormalities, and infectious disease (Supplementary Table S6).

**Unique gene classifiers distinguish individual experimental cohorts**

To determine how each group individually contributed to this global signature, we proceeded to identify classifiers that could discriminate each group from the others using only these 632 genes (Supplementary Figs. S3 and S4). Every group was compared with the other two groups separately ($P < 0.05$; the t test) generating gene lists that could distinguish either control mice from those irradiated with an acute dose, control mice from those irradiated with a fractionated dose, or mice irradiated with an acute dose versus a fractionated dose (199, 317, and 136 genes, respectively; Supplementary Fig. S3). Overlapping genes between the two comparisons in which an individual group was tested defined the classifier for that particular group (Supplementary Figs. S3 and S4D–S4L). This culminated in classifiers consisting of 76 genes for control K-ras$^{LA1}$ mice, 20 genes for K-ras$^{LA1}$ mice irradiated with an acute dose, and 45 genes for K-ras$^{LA1}$ mice irradiated with a fractionated dose. HCL, PCA, and k-means clustering verified the capacity of these classifiers to isolate the associated experimental group from the others (Supplementary Fig. S4D–S4L).

**Figure 2.** Tumor burden and grade in irradiated K-ras$^{LA1}$ lungs comparable with unirradiated controls 70 days after irradiation. A to C, representative scans of H&E-stained K-ras$^{LA1}$ lungs 70 days after irradiation with an acute or fractionated dose of 1.0 Gy $^{56}$Fe-particles and age-matched unirradiated controls demonstrate low tumor burden. D to I, representative images of 70 days lungs indicates lesions composed of only benign lung hyperplasias (D–F) or adenomas (G–I). Inset higher magnification of lesion. J to M, quantification of overall number and size of hyperplasias (J and K) and adenomas (L and M) in K-ras$^{LA1}$ lungs from 40 to 100 days after irradiation with an acute or fractionated dose of 1.0 Gy $^{56}$Fe-particles and age-matched unirradiated controls ($N = 6–8$ mice/group/time point); 3 sections/mouse quantified). Scale bars, 100 μm; graphs, mean ± SEM; tumor differences determined by two-way ANOVA with Tukey correction; *, $P < 0.05$. 

unirradiated control K-ras$^{LA1}$ lungs (Supplementary Fig. S4A). This allotted sufficient time for irradiated lungs to recover from any early radiation damage while short enough that tissues/tumors from all cohorts remained histologically identical (Fig. 2). At this time point, no advanced adenomas or carcinomas were observed in either control or irradiated mice and inflammatory infiltrates, such as lymphocytes and macrophages, were negligible (Fig. 2). Thus, any molecular changes observed would be predicted to be directly associated to changes in the evolving carcinogenic process.

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Although experimental groups could not be distinguished histologically, collective differences in gene expression were sufficient to segregate these groups by either hierarchical clustering (HCL) or principal component analysis (PCA) using 632 genes (Supplementary Figs. S3, S4B and S4C). The large number of genes required for this segregation further signifies the overwhelming complexity inherent to the genetic analysis of multicellular tissues that do not have overt histologic differences. Interrogation of the underlying biology encompassed by this gene signature with Ingenuity pathway analysis (IPA) revealed that these genes could be ascribed to several networks associated with cancer, organismal injury and abnormalities, and infectious disease (Supplementary Table S6).
"Fractionated" classifier capable of predicting lung cancer patient survival

Identification of a unique classifier for each experimental cohort indicated that these groups had already begun to diverge at the molecular level 70 days after irradiation. It is important to note that the relevance of these classifiers to the observed biologic phenotypes is based on the static time point from which they were derived and thus may be limited in their general applicability to understanding how radiation exposure impacted lung carcinogenesis (2, 4).

Increasingly, microarray profiling has been performed on primary human tumors at various stages and from different tissues, which are then consolidated into large databases replete with associated clinical and survival data (19, 20). This has enabled data mining for gene signatures correlated with patient survival in an attempt to refine clinical prognoses (19, 20). Because each of our experimental groups could be stratified on the basis of the incidence of invasive lung adenocarcinoma and survival (Fig. 1F and G), we hypothesized that our early-stage classifiers would differentially predict patient survival if the captured gene expression was truly indicative of the resultant phenotypes. Classifiers were, thus, used to create prediction models with three independent human lung cancer microarray datasets, including two publicly available datasets (19, 20) and our University of Texas (UT)—Lung NCI SPORE dataset.

Consistent across all three datasets, only the "fractionated" classifier retained the capacity to predict overall patient survival when prediction models were built using either PCA or k-means clustering (Fig. 3 and Supplementary Fig. S5). Addition of the 45-gene "fractionated" classifier to clinical covariates improved survival prediction compared with using clinical criteria alone [$P = 0.0001$ (classifier + covariates) vs. $P = 0.0028$ (covariates alone); the log-rank test; SPORE dataset]. Interestingly, these analyses also demonstrated that the mutational status of K-ras is not correlated with survival and this correlation is not improved by the addition of the "fractionated" classifier ($P = 0.16$ vs. $P = 0.66$; Cox analysis).

Figure 3. "Fractionated" classifier retains capacity to predict overall lung cancer patient survival across multiple human lung cancer datasets. A to I, prediction models constructed with PCA using gene classifiers that identify unirradiated K-rasLA1 control mice (A–C) or those irradiated with an acute (D–F) or fractionated dose of 1.0 Gy $^{56}$Fe-particles (G–I) and three independent human lung adenocarcinoma datasets. Results demonstrate that only "fractionated" classifier is capable of predicting patient survival and these results are independent of dataset specificity. J and K, cross-validation of prediction model constructed with "fractionated" classifier. Prediction model was constructed with PCA using "fractionated" classifier and SPORE lung adenocarcinoma dataset and then model was tested in either NCI or Aichi adenocarcinoma dataset (J and K, respectively). Red lines denote high-risk patients and black lines denote low-risk patients. Survival differences were determined by the log-rank test. HRs and 95% CIs are relative to high-risk patients.
To verify that our 45-gene classifier was nonrandom, bootstrap analysis \((n = 1,000)\) was performed using gene sets containing 45 genes randomly selected from the 11,051 genes in common between the three lung cancer datasets. Less than 1% of these random datasets performed comparably or better than our 45-gene “fractionated” classifier within the SPORE dataset. None of the random gene sets were capable of performing in all three datasets.

**Inflammation-related signaling networks differentially activated across experimental groups**

The capacity of the “fractionated” classifier to predict overall survival in patients with lung cancer demonstrated that these 45 genes retained clinical relevance in humans. IPA annotations of this classifier indicated that these genes are highly associated with networks related to cancer, organismal injury and abnormalities, and infectious disease (Fig. 4A–D and Supplementary Table S6). To determine whether these networks were differentially expressed between our experimental groups, we analyzed the activation status of several integral or related network proteins in whole lungs from K-ras\(^{\text{LA1}}\) mice 70 days after irradiation. Most notable was the coincident increase in the activation of the protooncogene JUN and the tumor suppressor mitogen-activated protein kinase 14 (MAPK14) p38 specifically in lung tissues from animals that were irradiated with an acute dose of radiation (Fig. 4E and G). The transcription factor, STAT3, is constitutively activated in many solid tumors and promotes tumor immunosuppression (21). STAT3 activation is mediated by several growth factors and cytokines, including those induced by oncogenic Ras, environmental stress, and tissue damage (21, 22). Phosphorylation of tyrosine residue 705 was increased in the lungs of both irradiated groups compared with unirradiated control K-
ras<sup>LA1</sup> lungs. The phosphorylation of serine residue 727, however, was slightly increased only in K-ras<sup>LA1</sup> lungs irradiated with fractionated dose (Fig. 4E and H). Activation of the canonical NF-κB pathway, which has been shown to be required for lung carcinogenesis (23, 24), was observed, but there was no difference between groups (Fig. 4E and I).

As the differential activation was detected in whole tissue extracts from K-ras<sup>LA1</sup> lungs, immunohistochemical analysis of p-cJUN and p-STAT3 Y705 was performed to determine which cells are responding in this fashion. Both activated signaling proteins were detected in the initiated lesions and in noninvolved type II cells and endothelial cells from the surrounding lung parenchyma (Fig. 5). Cells contained within the margins of hyperplasias and adenomas, however, were those in which an activation differential was detected (Supplementary Fig. S5S and S5T). This apparent disparity in key inflammation-related networks between experimental groups suggested that radiation exposure affected lung cancer progression by altering the local inflammatory responses and this affect was dependent on the dose regimen.

**Inflammation-based "fractionated" classifier relevant to breast cancer patient survival**

Lung cancer is a heterogeneous disease consisting of several subtypes. Activation of mutant K-ras G12D in K-ras<sup>LA1</sup> mice specifically results in the formation of adenocarcinoma, which is the most common form of lung cancer in humans (25). Inflammation has, however, been associated with several types of cancer, including breast and squamous cell carcinoma (SCC; ref. 5). As large-scale, annotated microarray datasets from primary human tumors are available for these cancer types, we proceeded to determine the global significance of our "fractionated" classifier by examining its relevance to these additional cancer types. Prediction models were constructed using our 45-gene "fractionated" classifier with several independent breast and squamous cell lung carcinoma datasets. Although the expression of these genes alone was not capable of predicting the survival of patients with squamous cell lung cancer, patients with breast cancer with a higher risk of mortality could be predicted in all three independent datasets (Fig. 6 and Supplementary Fig. S6). Therefore, identification of a unique inflammation-related classifier capable of segregating K-ras<sup>LA1</sup> mice irradiated with a fractionated dose of radiation, which have an increased incidence of invasive carcinoma, has revealed a potential intimate relationship between the disruption of specific inflammation processes and the survival of human patients with both lung and breast adenocarcinoma.

**Data reduction retains predictive capacity and implicates TNF as a potential driver**

Although our 45-gene "fractionated" classifier demonstrated utility in multiple datasets derived from cancers of disparate tissues, the power of this classifier is reduced due to the complexity introduced by the overall number of genes. This is evident in the results from IPA network...
analysis as these genes can only be reduced to three non-overlapping networks with various associated functions (Fig. 4A and Supplementary Table S6). To minimize this complexity, we determined which genes within this classifier were most associated with human lung cancer survival. Univariate Cox regression analysis \((P < 0.01)\) of the 45 classifier genes using only one of the lung cancer microarray datasets, the University of Texas (UT)—Lung NCI SPORE dataset, resulted in six genes, \(\text{CHEK1, FANCI, FCGRT, MAGEB2, POLQ, and TMPO}\), that highly correlated with survival in "fractionated" classifier. Of these genes only \(\text{MAGEB2}\) was not contained within the first network predicted by IPA (Fig. 4A). Condensing the "fractionated" classifier in this fashion demonstrates that this classifier is 3.5-fold enriched for genes that are highly correlated with overall survival \((P < 0.01)\) when compared with the entire SPORE microarray dataset \((13.3\% \text{ vs. } 3.8\%; P < 0.01; \text{univariate Cox})\). In fact, only 2.8\% of the gene sets comprised of forty-five genes randomly selected from the SPORE dataset, have six genes or more associated with survival. Univariate Cox analysis \((P < 0.01)\) of the 11,051 genes in common between the three lung cancer datasets reveals 576 genes significantly associated with survival in the SPORE dataset. The six genes most correlated with survival from these 576 are not predictive in all three datasets when tested as predictive signatures. Bootstrap analysis \((n = 1,000)\) using gene sets comprised of six genes randomly selected from the 576 survival-associated genes revealed that only five of these gene sets perform comparably or better in all three lung cancer datasets. One of these five "retrospective"
gene sets was also predictive in all three breast cancer datasets at a level comparable with our “prospective” six-gene signature. There are no overlapping genes between those derived from the 45-gene classifier and these five random gene sets.

IPA analysis of the condensed “fractionated” classifier further suggests that the complexity inherent to the 45-gene classifier was reduced, as only one network could be predicted. This network is associated with infectious disease, inflammatory response, respiratory disease, and immunologic disease and is affected by the cytokine TNF (Fig. 6F and Supplementary Table S3). Validation that this data reduction did not alter the significance of the "fractionated" classifier for predicting overall human cancer patient survival was demonstrated by constructing prediction models using only these six genes and testing all lung and breast cancer datasets (Fig. 6B–K and Supplementary Fig. S7).

Discussion

These studies provide robust evidence that radiation exposure can enhance lung cancer progression in vivo. The breadth and scope of our experiments permitted evaluation of several endpoints and the influence of confounding variables such as genetic background and gender on these endpoints (Supplementary Tables S1–S5). Of particular interest is the observation that the incidence of invasive adenocarcinoma is increased following a fractionated dose of 56Fe-particles when compared with that following an equivalent acute dose or in unirradiated K-rasLA1 mice (Fig. 1F and Supplementary Table S3). When combined with the increased incidence observed following an acute lower dose of 34Fe-particles (Fig. 1F and Supplementary Table S3), this supports the controversial concept of an inverse dose-rate effect for carcinogenesis following high-energy radiation exposure (11).

Exploitation of the increased statistical power provided by the differential effect of dose fractionation on lung cancer progression enabled us to identify a genomic signature in whole lung that is driven by alterations to key inflammation-related pathways (Figs. 5A and 6F; Supplementary Table S6). Inflammation has a significant impact on the carcinogenic process as both a tumor suppressor and promoter and radiation exposure can induce inflammatory responses that can elicit both these effects (5, 6, 26). For instance, tumor eradication is enhanced if radiation exposure results in antitumor immunity (6). Radiation-induced inflammation, in contrast, may also result in late normal tissue damage and/or secondary malignancies thereby restricting its therapeutic potential for cancer treatment (3, 11, 26). These responses are dose-dependent and mediated in part by the differential activation of interconnected signaling pathways by oxidative stress, growth factors, and cytokines (6, 26). Subtle variations in one or more of these pathways dictate the elicited response. Among these pathways are those that we found to be disproportionately perturbed in tissues with no overt histologic differences (Supplementary Fig. S4 and 5E–I). Incorporation of the resultant phenotypes suggests that radiation exposure altered the normal inflammatory response in emerging tumors and that the inflammatory signature observed following a fractionated dose of radiation is permissive for tumor progression.

The contribution of inflammation on carcinogenic process has also been demonstrated in other tissues. Oncogenic KRAS mutations are frequently observed in human pancreatic cancer (27) and inflammation cooperates with oncogenic KRAS in the transformation of adult pancreatic acinar cells (28, 29). Furthermore, irradiated fibroblasts increase the invasive capacity of pancreatic cancer cells when cocultured in vitro through the secretion of the hepatocyte growth factor, which has an anti-inflammatory and antifibrotic effect in vivo (8, 30).

The identification of a gene classifier that predicts overall survival for human patients with lung and breast cancer suggests that the inflammatory dysregulation represented by this classifier critically affects carcinogenesis in both tissues. Data reduction by examining the genes that most correlate with survival within our “fractionated” classifier implies an essential role for TNF and the ubiquitylation system for regulating the observed phenotypes (Fig. 6F). This is in concordance with the disrupted signaling networks observed in K-rasLA1 lungs as these are affected by TNF stimulation (Fig. 5 and Fig. 6F). Ubiquitylation of several key proteins within the TNF signaling network modulates the resultant cellular response and there are many E3 ubiquitin protein ligases within the 632 genes that distinguish our experimental groups (Fig. 3B and C and Supplementary Table S6; ref. 31). F-box protein 42 (FBXO42), which regulates TP53, is the only E3 ubiquitin protein ligase unique to the “fractionated” classifier and is expressed at higher levels in animals that received the fractionated dose (32). The role of FBXO42 in the TNF response is currently not known.

Several other genes within our “fractionated” classifier provide further support for a role of an altered TNF response, including Toll-like receptor 1 and Wnt5a. Both genes are expressed at lower levels in the lungs of K-rasLA1 mice irradiated with a fractionated dose compared with the other two groups (Fig. 5A). In addition, the tumor suppressor Scribbled planar cell polarity protein (Scrib) was also expressed at lower levels in K-rasLA1 lungs following a fractionated dose of radiation (Fig. 5A and B). TNF stimulation induces cell death in cells expressing oncogenic KRAS or in which SCRIB expression is lost (33, 34). SCRIB deficiency in cells expressing oncogenic KRAS, however, subverts the TNF response to stimulate invasion (35). Finally, loss of SCRIB in mammary epithelia cooperates with c-Myc in the suppression of apoptosis and enhancing cellular transformation (36). How dose fractionation resulted in the downregulation of these genes requires further study.

Overall, we have demonstrated that radiation exposure may lead to several biologic effects in which severity may be dependent on radiation dose and quality, and radiation-induced inflammation seems to modulate these effects.
Derivation of a genetic signature capable of predicting patient survival with lung or breast cancer suggests explicit similarities between these tissues in their inflammatory responses to emergent tumors. Therefore, evaluation of these responses before treatment, in particular those related to TNF, could potentially assist lung and breast cancer patient stratification.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: O. Delgado, K.G. Batten, A.F. Gazdar, M.D. Story, J.D. Minna, J.W. Shay
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Delgado, J.A. Richardson, A.F. Gazdar, A.A. Kaisani, C. Behrens, M.D. Story, I.I. Wistuba
Writing, review, and/or revision of the manuscript: O. Delgado, K.G. Batten, L. Girard, W.E. Wright, M.D. Story, I.I. Wistuba, J.D. Minna, J.W. Shay

References
# Clinical Cancer Research

## Radiation-Enhanced Lung Cancer Progression in a Transgenic Mouse Model of Lung Cancer Is Predictive of Outcomes in Human Lung and Breast Cancer

Oliver Delgado, Kimberly G. Batten, James A. Richardson, et al.


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