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LKB1 mutations in NSCLC are associated with KEAP1/NRF2-dependent radiotherapy resistance targetable by glutaminase inhibition

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Abstract

Purpose: Radiotherapy (RT) with or without chemotherapy is a mainstay of treatment for locally advanced non-small cell lung cancer (NSCLC), but no predictive markers are currently available to

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Conflicts of interest statement

J. Zhang reports research funding and personal fees from BMS, Johnson and Johnson, AstraZeneca, Genepius, OrigMed, Innovent and Merck, outside the submitted work; J. Roth reports consultant; scientific advisor; ownership interest; Inventor on intellectual property licensed by Genprex; PI on Genprex sponsored research; J.V. Heymach reports receiving research support from AstraZeneca, Spectrum Pharmaceuticals, and Boehringer Ingelheim and has served on advisory committees for AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Catalyst, EMD Serono, Foundation Medicine, Hengrui Therapeutics, Genentech, GSK, Guardant Health, Eli Lilly, Merck, Novartis, Pfizer, Roche, Sanofi, Seattle Genetics, Spectrum, Takeda, and receives royalties and licensing fees from Spectrum. The remaining authors have declared that no conflict of interest exists.

select patients who will benefit from these therapies. In this study we investigated the association between alterations in *STK11/LKB1*, the second most common tumor suppressor in NSCLC, and response to radiotherapy as well as potential therapeutic approaches to improve outcomes.

Experimental design: We conducted a retrospective analysis of 194 patients with stage I-III NSCLC including 164 stage III patients bearing mutant or wild-type *STK11/LKB1* treated with RT, and assessed loco-regional recurrence (LRR), distant metastasis rates, disease-free survival (DFS), and overall survival (OS), and we investigated the causal role of LKB1 in mediating RT resistance using isogenic pairs of NSCLC cell lines with LKB1 loss or gain.

Results: In Stage III patients, with 4 years median follow-up, *STK11/LKB1* mutations were associated with higher LRR ($P = 0.0108$), and shorter DFS (HR 2.530, $P = 0.0029$) and OS (HR 2.198, $P = 0.0263$). LKB1 loss promoted relative resistance to RT which was dependent on the KEAP1/NRF2 pathway for redox homeostasis. Suppression of the KEAP1/NRF2 pathway via KEAP1 expression, or pharmacologic blockade of glutaminase (GLS) 1 sensitized LKB1-deficient tumors to RT.

Conclusions: These data provide evidence that LKB1 loss is associated with local-regional recurrence and poor clinical outcomes in NSCLC patients treated with RT and that targeting the KEAP1/NRF2 pathway or GLS inhibition are potential approaches to radiosensitize LKB1-deficient tumors.

Keywords

Non-small cell lung cancer; *STK11/LKB1*; NRF2; KEAP1; Radio-resistance

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, estimated to be responsible for nearly 1.76 million deaths annually (1). Among all lung cancer cases, greater than 80% are classified as non-small cell lung cancer (NSCLC). For patients with locally advanced NSCLC, radiotherapy (RT) alone or in combination with chemotherapy and adjuvant durvalumab are mainstays of treatment (2,3). However, loco-regional recurrence after radiotherapy remains a major clinical challenge, and biomarkers, other than *KEAP1* (4), *TP53* or *KRAS* (5-8), predicting treatment response have yet to be identified. Understanding the mechanisms underlying radio-resistance in NSCLC is critical for identifying novel effective therapeutic strategies and developing predictive biomarkers to select patients likely to benefit from these therapies.

The Liver kinase B1 (LKB1) protein, encoded by the *STK11* gene, is the second most commonly altered tumor-suppressor in NSCLC, with mutations or genomic loss occurring in 17-23% of NSCLC cases (9-11). LKB1 mutations are typically loss-of-function and frequently co-occur with *KRAS* activating mutations (12). *KRAS*-mutant tumors with co-occurring mutations in LKB1 (KL) are associated with an aggressive clinical course, shorter survival rates, and an immunosuppressed phenotype (13-15), and to date there are no validated therapeutic strategies for this tumor subtype. We and others have demonstrated that loss of LKB1 impacts tumor progression through alterations in energy metabolism, cell motility, cytokine suppression, and tumor immune suppression (16-20). LKB1 mutant

NSCLC tumors are also enriched for *KEAP1* mutations or bi-allelic loss. KEAP1 is a negative regulator of NRF2, a transcription factor that binds to Antioxidant Response Elements (ARE) on the DNA and initiates the transcription of a number of genes involved in regulation of redox balance and cellular detoxification (21). The KEAP1/NRF2 pathway is an established mechanism of radio-resistance in many cancers, including lung cancer due to enhanced expression of reactive oxygen species (ROS) scavengers and detoxification pathways in tumor cells with increased NRF2 (4,22-25). In NSCLC, LKB1-deficient tumors with *KEAP1* mutations express higher levels of NRF2-regulated genes (14,26), and KEAP1/NRF2 mutations were predictive of local recurrence after RT in patients with NSCLC (4).

Recent findings indicate that loss of LKB1 can induce metabolic changes leading to increased ROS production, while loss of KEAP1 expression can protect against ROS-mediated damage (18,27,28). Radiation induces ROS generation within cells, and inadequate removal of ROS results in oxidative stress and DNA damage resulting in cell death, whereas increased expression of antioxidant enzymes or of the presence of free radical scavenger results in lower ROS levels and radio-resistance (29,30). Preclinical reports have suggested that loss of LKB1 in *KRAS*-driven lung adenocarcinoma may render tumor cells less responsive to radiation (31), but the impact of LKB1 loss in NSCLC patients receiving radiotherapy is unknown.

Glutamate is a precursor for glutathione (GSH) synthesis, which is the major cellular antioxidant for redox homeostasis. Enhanced NRF2 expression protects tumor cell DNA from ROS-mediated damage, in part through NRF2-mediated upregulation of glutamate-cysteine ligase (GCLC), the enzyme that catalyzes the production of GSH from glutamate (32). Because of this “glutamine addiction”, glutaminase (GLS), the rate-limiting enzyme that converts glutamine to glutamate, has emerged as a potential therapeutic target (33). We recently reported that alterations in LKB1 and KEAP1 cooperatively drive metabolic adaptations in tumor cells that result in glutamine dependence and vulnerability to glutaminase inhibition in *KRAS* mutant NSCLC (18). Although the inhibition of glutamine metabolism has been reported as an effective radiosensitizing strategy in lung tumor cells (34), identification of useful biomarkers for predicting therapeutic response to this treatment remain a clinical need.

Here, we report that mutations in *STK11/LKB1* are a predictive marker for both local and distant recurrences in NSCLC patients treated with RT. Furthermore, we show that LKB1 loss promotes resistance to ionizing radiation and radiation-induced intracellular ROS generation at least in part due to a KEAP1/NRF2 pathway-dependent mechanism that can be targeted via GLS inhibition. Thus, targeting the KEAP1/NRF2 pathway, or glutamine metabolism through GLS inhibitors, are two potential approaches to radiosensitize LKB1-deficient NSCLC tumors.

Materials and methods

Patients

We queried the MD Anderson Lung Cancer Moon Shot GEMINI Project, a prospective database for patients with lung cancer incorporating collection of molecular profiling and

clinical information, for NSCLC patients undergoing definitive radiation treatment. Patients treated with neoadjuvant chemo-radiation who underwent surgery were not included in the analysis. This project is approved by the MD Anderson Cancer Center Institutional Review Board, and all patients signed informed consent form. This project was conducted in accordance with ethical guidelines including Declaration of Helsinki and U.S. Common Rule. Mutation status was determined using one of the next generation sequencing panels of 50, 134 or 409 genes used for routine clinical care at MD Anderson Cancer Center.

Cell culture

The four NSCLC human cell lines (A549, H460, H23 and Calu6) were purchased from the ATCC (Rockville, MD, USA) and maintained in RPMI-1640 with 10% FBS, 1% glutamine and 1% penicillin-streptomycin. Isogenic pairs were established by stable expression of wild-type LKB1 cDNA in the naturally LKB1-deficient A549 and H460 cell lines and stable shRNA-mediated LKB1 knockdown in Calu-6 were achieved by lentiviral transduction as previously reported (18).

Fingerprinting and mycoplasma test using MycoAlert mycoplasma detection kit (Lonza, Basel, Switzerland) were performed periodically to authentication. For *in vivo* injection cells were used within 10 passages from thawing. Cell lines were disregarded after 20 passages from thawing.

Western blotting

Western blot analysis of whole cell extracts was performed as previously reported (18). The following primary antibodies included LKB1 (D60C5) (1:1000, #3047, Cell Signaling Technology), KEAP1, (1:1000, #10503-2-AP, Proteintech), NQO1 (A180) (1:1000, #3187 Cell Signaling Technology), NRF2 (EP1808Y) (1:1000, ab62352, Abcam), GCLC (1:1000, ab41463, Abcam) and β -actin (1:5000, Abcam) were used.

Clonogenic survival assays

The radiation response and the effect of CB-839 as radio-sensitization was assessed in vitro using clonogenic survival assays. Cells were seeded in duplicates at three dilutions into 6-well plates and incubated for 12-16 hours prior to treatment. The cells were treated with either CB-839 (10 nmol/L) or DMSO for 4 h prior to irradiation. The cells were then irradiated at doses from 0 to 6 Gy using a JL Shepherd Mark I-68A with ^{137}Cs source at a dose rate of 3.254 Gy/minute. The medium was then replaced by fresh medium and cells maintained in normal cultural conditions for 10-14 days. The colonies were stained by crystal violet (0.5% w/v). Colonies containing at least 50 cells were counted. The surviving fraction was calculated as previously reported (18,35) using SigmaPlot 10.0 (San Jose, CA, USA). The assays were repeated 3 times for each cell line.

Measurement of ROS levels

Cells were treated with either DMSO or CB-839 (1 $\mu\text{mol/L}$) for 4 h followed by irradiation (8 Gy). Intracellular ROS levels were measured 72 hours after irradiation using 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA) (Sigma). The cells were incubated with DCFDA (20 $\mu\text{mol/L}$) for 30 min at 37°C. Fluorescence of oxidized DCF was measured at an

excitation wavelength of 488 nM and an emission wavelength of 535 nM for the DCFDA using a FAC Scan flow cytometer (BD Biosciences, San Jose, CA, USA).

Flow cytometry apoptosis assay

Cells were treated with either DMSO or CB-839 (1 μ mol/L) for 4 hours with or without irradiation (8 Gy) as described above. Cells were then collected and stained with PE conjugated AnnexinV and 7-AAD according to the manufacturer's instruction. The flow cytometry was analyzed using a FAC Scan flow cytometry (BD Biosciences, San Jose, CA, USA). The assays were done in triplicate.

Drugs

CB-839 was provided by Calithera Biosciences (EMD Millipore, Billerica, MA). CB-839 were re-suspended in DMSO to a final concentration of 10mM. Drug aliquots were stored at -80°C and each aliquot was used only once.

In vivo studies

Athymic female nude mice 6-8 weeks old from (Invigo, Indianapolis, IN, 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 (approved protocol numbers; 00001213-RN02 and 00001217-RN02). For A549 xenografts, mice were subcutaneously inoculated with a total of 7×10^6 A549-CTR, A549-LKB1 and A549-KEAP1 cells into their right hind legs. When the tumor reached 250-300 mm^3 , mice were randomized into different treatment groups (7-8 animals per group). Mice were irradiated with 5 Gy of gamma irradiation by using a Mark 1-68A cell irradiator with a Cesium-137 source at a dose rate of 3.254 Gy per minute (J.L. Shepherd & Associates, San Fernando, CA) to deliver local irradiation to the flank or thigh of lead-shielded mice. Two axes of the tumor (L, longest axis; W, shortest axis) were measured three times per week after IR using Vernier calipers. Tumor volume was calculated using the formula: $(L \times W^2) / 2 (\text{mm}^3)$. The tumor growth curve was calculated using GraphPad Prism 6.

Statistic

Continuous and ordinal variables were summarized as median and range, categorical variables were reported as counts and percentages. The relationship between variables was assessed with χ^2 or Fisher exact test, as appropriate. Outcomes were analyzed in terms of loco-regional recurrence (LRR), distant metastases (DM), disease free survival (DFS) and overall survival (OS). LRR was defined as previously described (36). DM was defined as disease recurrence in a different lobe or failure outside the chest. Time-to-failure endpoints were calculated from the date of diagnosis to the date of the first disease recurrence. DFS was calculated from the date of diagnosis to the date of the first disease recurrence, development of second lung primary malignancy, or death from any cause. Second primary lung malignancy was defined as development of a lung nodule that did not meet radiological

criteria for metastasis, or that was biopsy proven to have distinct histologic subtype (adenocarcinoma vs squamous cell carcinoma). OS was calculated from the date of diagnosis to the date of death from any cause or last follow-up. Patients who did not develop an event were censored at the date of last follow-up. The survival probability was computed using the Kaplan-Meier method and heterogeneity in survival rates among strata was assessed using the log-rank test. In the presence of competing risks (death) when performing survival analyses for any disease recurrence, an alternative cumulative incidence competing risk method was used to overcome the overestimated probabilities of disease recurrence. Time to event endpoints were calculated with the Kaplan-Meier method and the log-rank test. Hazard ratios (HRs) and corresponding 95% confidence intervals (95% CI) were calculated according to the Wald method. P value ≤ 0.05 was considered as statistically significant.

Results obtained from *in vitro* and *in vivo* analysis were shown as means $\pm$ SD or SEM of three or more independent experiments. Data were analyzed using unpaired Student's t -test, Mann-Whitney U test or one-way ANOVA. All P values are two-tailed and for all analyses, $P \leq 0.05$ is considered statistically significant, unless otherwise specified. Wilcoxon analysis was performed to determine association of mutation status and radiation sensitivity. All statistical analyses were conducted using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA), SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and R version 3.3.3.

Results

STK11/LKB1 mutation is associated with worse outcome after definitive radiation in patients with stage III NSCLC

To investigate the association between *STK11* mutations and outcome after radiotherapy in NSCLC, we examined 194 consecutive patients consented for the GEMINI database at MD Anderson Center who had undergone molecular profiling and received definitive radiation treatment from March/2007 until April/2017 (Table S1). The median age of the study population at diagnosis was 64 years, with the majority of patients being female (53.6%). Most patients had a good performance status (PS 0-1: 94.5%) and were current (15%) or former (67.4%) smokers. The most common histology was adenocarcinoma (71.5%), 9.3% presented squamous histology and the vast majority had stage III disease (84.5%). We focused the analysis on stage III patients treated prior to the standard use of durvalumab as maintenance therapy so that local-regional recurrence (LRR) and disease-free survival (DFS) would not be confounded by maintenance durvalumab treatment, given the association between *STK11* and response to PD-1/PD-L1 inhibition (37).

We assessed the cohort with stage III disease treated with definitive radiation ($N=164$), with or without concurrent chemotherapy (Table 1). After a median follow-up of 4.0 years (range: 0.3-13.1 years), the presence of *STK11*/LKB1 mutation was associated with significantly higher cumulative loco-regional recurrence (LRR) rates ($P = 0.0108$; Table 2; Figure 1A), shorter disease free-survival (DFS) (HR 2.53, 95% CI 1.375-4.657, $P = 0.0029$, Cox regression model; Table 3; Figure 1B) and overall survival (OS) (HR 2.198, 95% CI 1.097-4.405, $P = 0.0263$, Cox regression model; Table 4; Figure 1C) than patients with

STK11/LKB1 wild type tumors. Distant metastasis (DM) failure rate was numerically higher in the *STK11/LKB1* mutant group compared to the *STK11/LKB1* wild type group but this was not statistically significant (1-year DM rate: 66.7% vs 39.3%, $P = 0.1129$; Table S2, Figure 1D). Estimated cumulative 1-year LRR rate using competing risk analysis was significantly higher in the *STK11/LKB1* group (25.0% [95%CI: 4.8-53.1] vs 10.8% [95%CI: 6.5-16.5], $P = 0.0108$, Table 2). In addition, 5-year DFS and OS rates were significantly lower in the *STK11/LKB1* group (5-yr DFS: 0% [95%CI: NA] vs 14.6% [95%CI: 8.9-21.6], $P = 0.0020$; 5-yr OS: 15.6% [95%CI: 1.1-46.4] vs 42.0% [95%CI: 32.7-51.0], $P = 0.0228$, Table S3). Of the 7 patients whose tumor harbored *STK11/LKB1* mutations and were censored for 1-year LRR competing risk analysis, 6 patients (85.7%) had subsequent LRR during their disease course. *STK11/LKB1* mutation status remained significantly associated with worse OS in a multivariate analysis including *STK11/LKB1* status and age (the two characteristics associated with OS in univariate analysis) (Table 5), and was the single variable associated with poor DFS in univariate analysis (Table 3). In a subgroup analysis of patients with stage III disease that received concurrent chemoradiation and had PS 0-1, 1-year LRR rate was also significantly higher for the *STK11/LKB1* group (27.3% [95%CI 5.2-56.6] vs 9.0% [95%CI 4.9-14.6], $P = 0.0023$) (Figure S1).

We further analyzed clinical outcomes among stage III NSCLC patients treated with definitive radiation according to *KRAS* subgroups. 51 of 164 patients (31.09%) had NSCLCs harboring a *KRAS* mutation. Of the 51 patients with NSCLC harboring a *KRAS* mutation, 7 patients (13.72%) had *STK11/LKB1* co-mutation (KL group), 20 patients (39.21%) had *TP53* co-mutation (KP group), and the remaining 24 patients (47.05%) had no *TP53* and no *STK11/LKB1* co-mutations (K group) (Figure 2A). Given the size of the cohorts, this analysis of *KRAS* subgroups had a limited statistical power. Nevertheless, it showed that patients with KL and KP tumors displayed shorter DFS and higher LRR rate compared to K tumors, but this was not statistically significant (5-year DFS: KL 0% vs. KP 10% vs K 27.3%, Kaplan-Meier $P = 0.0701$; 1-year LRR rate: K 8.3% [95%CI 1.4-23.7] vs KL 14.3% [95%CI 0.3-53.1] vs KP 10.5% [95%CI 1.9-33.7], $P = 0.2983$). Pairwise comparison of patients with KL vs KP tumors revealed statistically significant shorter DFS in KL patients ($P = 0.0211$) (Figure 2B&C; Tables 2, 3, and S3). No differences were found for DM rate or OS for KL vs KP tumors (Figures S2A&B). Because *KRAS* mutations are rare in squamous NSCLC, we then excluded patients with squamous cell carcinoma (SCC) from our analysis. 3 of the 51 (5.88%) *KRAS* mutant NSCLCs revealed a squamous histology. Of the 48 patients with non-squamous NSCLC harboring *KRAS* mutation, 7 patients (14.58%) harbored KL tumors, 17 patients (35.41%) KP, and the remaining 24 patients (50.00%) K tumors (Figure S3A and Table S4). Likewise, patients with KL tumors and KP tumors showed numerically shorter DFS and higher LRR rate compared to K tumors, but this was not statistically significant (5-year DFS: KL 0% vs. KP 5.9% vs K 27.3%, Kaplan-Meier $P = 0.0843$; 1-year LRR rate: K 8.3% [95%CI 1.4-23.7] vs KL 14.3% [95%CI 0.3-53.1] vs KP 12.5% [95%CI 1.9-33.7], $P = 0.3573$). DFS for patients harboring KL tumors was significantly shorter when compared with KP patients, $P = 0.0462$ (Figure S3B&C; Tables S5 and S6).

To better dissect the impact of *KRAS* and *TP53* mutations on clinical outcome from radiation therapy, we also performed subgroup analysis of tumors harboring co-occurring

KRAS and *TP53* mutations (KP tumors) vs tumors harboring *KRAS* or *TP53* wild-type (non-KP tumors), and showed no difference in 1-year LRR rate (10.5% [95%CI 1.6-29.1] vs 12.1% [95%CI 7.3-18.1], $P = 0.9026$) (Figure S4A and Table 2). Comparison of clinical outcomes for patients whose tumors harbored a *KRAS* mutation (*KRAS* Mut) vs patients whose tumors were *KRAS* wild-type (*KRAS* WT) showed no difference in 1-year LRR rate (10.0% [95%CI 3.6-20.3] vs 12.8% [95%CI 7.3-19.8], $P = 0.4408$) (Figure S4B). Finally, we compared 1-year LRR rates between three groups: patients whose tumors harbored a *STK11/LKB1* mutation (*STK11* Mut), patients whose tumors harbored co-occurring *KRAS* and *TP53* mutations (KP), and patients whose harbor *KRAS* or *TP53* or *STK11* wild-type tumors (Other) (Figure S4C). In this comparison we observed that the *STK11* mutant subgroup had significantly higher 1-year LRR rate compared to KP and all other genotype subgroups (*STK11* Mut 25% [95%CI 4.8-53.1] vs KP 10.5% [95%CI 1.6-29.1] vs other 10.9% [95%CI 6.2-17.0], $P = 0.0379$). *STK11* Mut vs other pairwise comparison showed significantly higher 1-year LRR rate ($P = 0.0143$), but not KP vs other or *STK11* Mut vs KP revealed significant differences (Figure S4C).

LKB1 loss drives radio-resistance in NSCLC tumor cells via activation KEAP1/NRF2 pathway

We previously reported that LKB1-deficient tumors are characterized by metabolic adaptations that result in glutamine dependence and upregulation of *NRF2* and its downstream target genes as a compensatory mechanism to maintain redox homeostasis (18). To investigate the impact of *STK11/LKB1* mutations over radiotherapy resistance *in vitro* we first screened a panel of thirty-three NSCLC cell lines for radiation sensitivity (Figure S5A). Consistent with clinical data, *STK11* and *KEAP1* but not *KRAS* or *TP53* mutations, correlated with radiation resistance (Figure S5B), although differences did not reach statistical significance due to a limited number of samples. To further explore radio-resistance associated to LKB1 loss, we then established three pairs of isogenic *KRAS*-mutant LKB1 deficient/proficient NSCLC cell lines by either re-expressing wild-type LKB1 in LKB1-deficient cells (A549 and H460) or stably expressing shRNA-targeting LKB1 in LKB1-proficient NSCLC cells (Calu-6). We previously reported that re-expression of LKB1 results in decreased expression of *NRF2* and *NRF2*-regulated genes, including NAD(P)H Quinone Dehydrogenase 1 (NQO1) in A549 and H460 cells, and knockdown of LKB1 in Calu-6 cells enhanced *NRF2* and NQO1 expression (18). To investigate the underlying mechanism by which LKB1 loss contributes to radiation resistance, A549 cells with or without LKB1 expression were exposed to ionizing radiation (IR) and effects on cell proliferation were determined by clonogenic survival assay (Figure 3A). LKB1 re-expression showed an increased radio-sensitivity with a DER (Dose Enhancement Ratio) of 1.4 at a surviving fraction of 0.5 compared to LKB1 deficient cells ($P = 0.0057$ at 2 Gy and $P < 0.0001$ at 4 and 6 Gy, and SD for control or LKB1 0Gy; 0.16 or 0.21, 2Gy; 0.15 or 0.17, 4Gy; 0.07 or 0.05 and 6Gy; 0.04 or 0.03 respectively (Figure 3B). Consistently, LKB1 overexpression in H460 cell lines resulted in enhanced radio-sensitivity compared to vector control cells, with a mean of the surviving fraction for LKB1 overexpressing H460 cells of 32%, compared to 55% for the H460 vector control cells at the 2 Gy IR dose ($P = 0.007$; Figure 3C). Similarly, knockdown of LKB1 increased the radio-resistance of the LKB1-proficient Calu-6 cell line. The surviving fraction was significantly higher in LKB1

knockdown Calu-6 cells (23.7%) compared to control cells (13.4%) ($P = 0.023$) when cells were treated with 4 Gy dose of IR (Figure 3D).

To determine whether the KEAP1/NRF2 pathway contributes to IR resistance induced by LKB1 loss, we stably expressed wild-type KEAP1 in A549 cells (LKB1 mutant/KEAP1-deficient). Re-expression of KEAP1 resulted in decreased expression of NRF2, NQO1 and glutamate-cysteine ligase catalytic subunit (GCLC) as determined by Western blotting (Figure S6A) and significantly enhanced radio-sensitivity compared to A549 vector control cells ($P < 0.0001$ at 2 and 4 Gy, $P = 0.01221$ at 6 Gy, SD for control or LKB1; 0Gy; 0.21 or 0.12, 2Gy; 0.13 or 0.15, 4Gy; 0.08 or 0.08 and 6Gy; 0.05 or 0.03 respectively; DER at a surviving fraction of 0.5 = 1.4) (Figures 3E&F). Similarly, depletion of KEAP1 increased the radio-resistance of H23 cells (Figure S6B), a KEAP1-proficient NSCLC cell lines. H23 control cells displayed greater sensitivity (1.6% of surviving fraction) to IR treatment (2 Gy) than H23 cells with knockdown of KEAP1 (7.4%), although this was not statistically significant (Figure S6B). Likewise, knock out of *Lkb1* (KL) and/or *Keap1* (KK or KKK) in a *Kras*-mutant murine cell line (LKR13) significantly enhanced radio-resistance compared with *Kras*-mutant control cells (K) (Figure S6C). Collectively, these data support that LKB1 loss promotes radio-resistance via a mechanism dependent, at least in part, on the KEAP1/NRF2 pathway in NSCLC tumor cells.

LKB1 loss drives radio-resistance by reducing IR-derived ROS generation in a NRF2-dependent manner

Given recent reports that LKB1 loss results in increased NRF2 expression which, in turn, reduces ROS levels (18), and since radiation-derived cytotoxic effects are mediated, at least in part, by induction of intracellular ROS (29,30), we next sought to determine whether loss of LKB1 or KEAP1/NRF2 activation protects tumor cells from ROS-mediated damage and contributes to radio-resistance. LKB1 and KEAP1 A549 isogenic pairs were irradiated at a dose of 8 Gy and intracellular ROS levels were measured after 72 hours (Figure 3G). As expected, re-expression of LKB1 reduced intracellular ROS levels, while KEAP1 knockdown significantly increased redox stress in non-treated cells. In all three cell lines, exposure to IR resulted in a rise in ROS levels. However, there was a significantly greater increase in ROS production following IR in LKB1 and KEAP1 expressing cells compared to A549 control cells lacking LKB1 and KEAP1 (Figure 3G). These findings suggested that the generation of ROS, as a result of IR exposure, is impaired in LKB1 and KEAP1 mutant cells and that this may contribute to the enhanced radiation resistance observed in LKB1 and KEAP1 deficient NSCLC cells. We next evaluated IR-induced cell apoptosis by annexin-V/7-AAD staining. In NSCLC A549 cells, IR exposure resulted in a reduced percentage of live cells from baseline in LKB1-proficient cells than in LKB1-deficient parental cells ($P = 0.0603$). A similar experiment was performed using stably expressed wild-type KEAP1 in A549 cells. NRF2 pathway suppression (via KEAP1 re-expression) significantly enhanced cytotoxic effect induced by IR ($P = 0.0164$) (Figure 3H). Taken together, our findings indicate that LKB1 and KEAP1 loss may protect tumor cells from the cytotoxic effects of IR due to a greater compensation of radio-derived intracellular ROS generation by activation of the KEAP1/NRF2 axis.

KEAP1 loss promotes radiotherapy resistance in KRAS/LKB1 mutant NSCLC xenografts models

We next sought to determine whether loss of LKB1 and/or KEAP1 impacts radiation sensitivity *in vivo*. Supporting *in vitro* data, radiation did not impair the growth of A549 (A549 control) tumors, which harbor co-mutations in *KRAS*, *STK11/LKB1*, and *KEAP1* genes (Figure 4A), there were not significant differences in progression free survival (PFS) rate (Figure 4B) between irradiated and control animals. In contrast, RT was modestly effective in A549 tumors with LKB1 re-expression (A549 LKB1) (Figure 4C), and no differences were observed in the survival rate between IR and control arms (Figure 4D). RT significantly impaired tumor growth in A549 KEAP1 expressing cells (A549 KEAP1) (Figure 4E) and prolonged the median PFS of A549 KEAP1 tumor-bearing mice ($P = 0.04$; Figure 4F). Collectively, these data provide additional evidence that loss of *STK11/LKB1* and more significantly, KEAP1 are potential mediators of radio-resistance *in vivo*.

Impairment of redox homeostasis by GLS inhibition confers radiation sensitivity in LKB1- and KEAP1-deficient cells

As previously described, enhanced NRF2 can protect LKB1-deficient cells from ROS-mediated damage in part by the NRF2-mediated up-regulation of GCLC enzyme that catalyzes the production of GSH from glutamate. Our previous reports indicate that conversion of glutamine to glutamate by GLS is a key step in the maintenance of redox homeostasis in LKB1-deficient tumors, particularly those with upregulation of the KEAP1/NRF2 pathway, and these tumors therefore display heightened sensitivity to GLS inhibition (18). Therefore, we next analyzed whether GLS inhibition could sensitize LKB1-deficient tumors to RT. We treated A549 cells with GLS inhibitors (also called CB-839) and then measured intracellular ROS levels 72 hours after IR treatment. GLS inhibition (1 μ M CB-839) markedly increased IR-induced ROS in A549 control cells (Figure 5A), while ROS induction was reduced when cells re-expressed LKB1 (Figure 5B) or KEAP1 (Figure 5C). Combination of 1 μ M CB-839 with IR significantly induced intracellular ROS production in A549 control cells when compared to IR alone (Figure 5A). Although IR treatment alone significantly increased ROS levels in A549-LKB1 cells, there were no greater induction of ROS levels when combined with GLSi (Figure 5B). In A549-KEAP1 cells, GLSi treatment plus IR resulted in a modest increase in ROS compared with IR alone, although this increase was far smaller than the increase induced by GLSi in the A549 control cells (Figure 5C). These data indicate that the GLSi-induced increase in ROS is at least in part dependent on the LKB1 and KEAP1 pathways.

We then explored whether inhibition of GLS enhanced IR cytotoxic effects in radio-resistant LKB1-deficient tumor cells. Consistent with previous redox induction data, treatment with 1 μ M CB-839 enhanced IR-induced apoptosis compared to control, CB-839 or IR alone in A549 parental cells, although differences did not reach statistical significance (p -value=0.119) (Figure 5D). Conversely, combined treatment of CB-839 and IR did not significantly increase apoptosis induction compared with IR-only-treated cells in A549-LKB1 (Figure 5E) nor A549-KEAP1 (Figure 5F) cells. We next treated A549 control, A549/LKB1 and A549/KEAP1 cells with CB-839 and evaluated sensitivity to IR by clonogenic survival assay (Fig. 5G&H). Quantification of surviving fractions (Figure 5I) revealed that

GLS inhibition strongly radio-sensitized A549 control cells (LKB1/KEAP1-deficient) with a DER at a surviving fraction of 0.5 of 1.8. Re-expression of LKB1 abrogated the CB-839-induced radio-sensitization with a DER at a surviving fraction of 0.5 were 0.8. Similar results were found in KEAP1 re-expressing cells after GLS inhibition (Figure 5I). As expected, GLS blockade significantly radio-sensitized KK and KLK cells with a DER at surviving fraction of 0.7 of 2.19 and 1.63 respectively. Conversely, CB-839 treatment did not enhance radiotherapy effects in K or KL cells (Figure S7). These data reveal that in LKB1 and/or KEAP1-deficient tumors, inhibition of glutamine metabolism using GLS inhibition impairs redox homeostasis and tolerance to oxidative stress, resulting in radio-sensitization of LKB1-deficient tumor cells.

Discussion

In this study we investigated the association between alterations in *STK11/LKB1*, the second most common tumor suppressor in NSCLC, and response to radiotherapy as well as potential therapeutic approaches to improve outcomes. We show that in patients with stage III, locally advanced NSCLC treated with RT, *STK11/LKB1* mutations were associated with significantly higher cumulative rates of loco-regional failure and shorter DFS and OS than tumors with wild-type *STK11/LKB1*. We next investigated the impact of LKB1 loss in preclinical models. *In vitro*, LKB1-deficient tumors displayed a radio-resistant phenotype, and modulation of LKB1 expression modified sensitivity to IR, supporting our clinical observations and providing evidence for a causative role of LKB1 in modulating response to RT.

We also investigate the mechanism(s) underlying the radioresistance induced by LKB1 loss. Prior studies have demonstrated that *STK11/LKB1* mutant tumors display metabolic alterations that render them sensitive to energetic and oxidative stress, and that upregulation of the KEAP1/NRF2 pathway (via KEAP1 loss or other mechanisms) in these tumors is critical to maintain redox homeostasis and cell survival (14,18). More broadly, overexpression of NRF2 has been found to be one of the mechanisms for chemotherapy and/or radiation resistance in lung cancer (21). Inhibition of NRF2 induced ROS generation and resulted in enhanced sensitivity to chemotherapy and radiation-induced cytotoxicity (22,24,32,38). Our data indicate that LKB1 deficient tumors are particularly dependent on the KEAP1/NRF2 pathway for radioresistance and that GLS inhibition is a potential therapeutic approach to overcome this resistance. However, we were unable to assess the impact of *KEAP1* alterations in this clinical cohort as *KEAP1* genetic alterations were not covered in our genomic profiling panel. Enhanced NRF2 activation protects tumor cells from ROS-mediated damage, in part by the NRF2-mediated up-regulation of GCLC enzyme that catalyzes the production of glutathione (GSH), one of the major antioxidant molecule, from glutamate. Interestingly, GSH plays an important role in cellular defense against radiation (39). Likewise, KEAP1 loss is associated with RT resistance in different tumor types (22,24,32,38). Consistent with this observation, our data showed that re-expression of KEAP1 sensitized tumor cells to ionizing radiation *in vitro* and in animal studies. We also showed that modulation of LKB1 expression alone was sufficient to activate NRF2-dependent antioxidant response.

We previously observed that the LKB1 and KEAP1/NRF2 pathway cooperate to promote enhanced dependence on glutamine metabolism and vulnerability to glutaminase inhibitor CB-839 (18). Here, we observe that LKB1-deficient tumors are vulnerable to therapies that target NRF2-mediated detoxification of ROS. With these protections removed, LKB1-deficient redox stressed tumors are also potentially vulnerable to ROS-mediated DNA damage. A growing body of evidence supports the idea that inhibition of GLS with small molecule inhibitors or by genetic knockdown leads to glutathione depletion, which leads to marked changes in response to radiation treatment (34,40,41). Here, we demonstrate that the GLS inhibition could sensitize LKB1-deficient tumor cells to ROS-mediated DNA damage induced by radiation, overcoming radio-resistance associated with *STK11*/LKB1 and/or KEAP1 mutations. Therefore, inhibition of critical NRF2 targets, such as GLS, may allow targeted radio-sensitization in patients with *STK11*/LKB1 mutations.

It is noteworthy that the retrospective clinical analysis presented here was from patients treated with RT prior to the routine use of the PD-L1 inhibitor durvalumab as maintenance therapy after chemoradiotherapy, based on the phase III PACIFIC study (42,43). Therefore, one limitation of the analysis is that the association between LKB1 and clinical outcome may be impacted by the inclusion of durvalumab. Previously, our group and others have reported that LKB1-deficient NSCLC are characterized by a markedly suppressed immune microenvironment within the tumor (14,44), increased expression of T cell exhaustion markers and tumor-promoting cytokines (19) and lack of response to PD-1/PD-L1 inhibitors (14,37,45). In light of this association between LKB1 loss and an immunosuppressed tumor microenvironment, it is noteworthy that the immune system plays an important role in prolonged response to RT (46,47). IR promotes an inflamed tumor microenvironment attracting the immune cells to the tumor site, which triggers innate and adaptive immune responses leading to tumor regression (48). While the preclinical studies here focused on cell-autonomous effects of LKB1 loss on RT response, it is possible that LKB1 loss may exert additional effects on RT response via tumor immunosuppression. Further studies using immunocompetent *in vivo* models are needed to further elucidate the contributions of cell autonomous vs immune-mediated mechanisms of RT resistance in LKB1-deficient tumors.

Although several prior studies have suggested a role of LKB1 expression in modulating response to radiotherapy based on preclinical experiments (31,49,50), none to our knowledge have examined the association of *STK11*/LKB1 mutations with radio-resistance in clinical data. We find that these alterations are associated with both LRR and shorter DFS/OS in patients treated with definitive RT. This analysis does have several limitations, including its retrospective nature, the limited sample size of the LKB1 cohort, which limits the statistical power of the analysis, and the fact that the analysis was conducted before the current standard of care changed to maintenance durvalumab as noted above. Also, it is noteworthy that TP53 mutations have been previously reported as a marker for predicting radiotherapy resistance in the clinic (5-8). In our cohort we observed a non-significant trend for shorter LRR and DFS for patients harboring KP mutations compared with K (*KRAS* mutant, wt *TP53*, wt *STK11*). In the KL subgroup, however, we observed a stronger association with radio-resistance in our pre-clinical and clinical analysis. Nevertheless, they do provide a foundation for further studies potentially targeting vulnerabilities associated with LKB1-deficient tumors. We find that RT resistance in LKB1-deficient tumors is

dependent on the KEAP1/NRF2 pathway in preclinical models, and we identify inhibition of glutamine metabolism with GLS inhibitors as a potential therapeutic strategy that merits further investigation for this highly refractory subgroup of NSCLC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

KEAP1	Kelch-like ECH-Associated Protein 1
LKB1	Liver Kinase B1
NRF2	Nuclear Factor Erythroid 2-like 2
IR	ionizing radiation
RT	radiotherapy

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Statement of Translational Relevance

Identification of predictive markers to select NSCLC patients who will benefit from radiotherapy (RT) treatments is a major clinical need. Here, we have conducted a retrospective analysis of 194 patients with stage I-III NSCLC undergoing definitive radiation treatment. We show that in patients with stage III, locally advanced NSCLC treated with RT, *STK11/LKB1* mutations were associated with significantly higher cumulative rates of loco-regional failure and shorter DFS and OS than tumors with wild-type *STK11/LKB1*. Furthermore, we show that LKB1 loss promotes resistance to ionizing radiation and radiation-induced intracellular ROS generation at least in part due to a KEAP1/NRF2 pathway-dependent mechanism that can be targeted via glutaminase (GLS) inhibition. Thus, targeting the KEAP1/NRF2 pathway, or glutamine metabolism through GLS inhibitors, are two potential approaches to radiosensitize LKB1-deficient NSCLC tumors.

Figure 1. LKB1 mutation status predicts radiation outcome in patients with stage III NSCLC. Estimated cumulative incidence curves illustrating (A) loco-regional recurrence (LRR) over time, (B) disease free survival (DFS), (C) overall survival (OS) by Kaplan-Meier analysis and (D) estimated cumulative incidence curves illustrating distant metastasis (DM) over time according to *STK11/LKB1* mutation status in patients with NSCLC treated with radiation.

Figure 2. Radiation outcome in KRAS mutant patients with stage III NSCLC.

(A) KRAS mutation subgroups. (B) Disease-free survival (DFS) across KRAS subgroups.

(C) Estimated cumulative incidence curves illustrating loco-regional recurrence (LRR) over time according to KRAS mutation subgroup in patients with NSCLC treated with radiation.

Figure 3. LKB1 loss contributes to radiation resistance by reducing IR-induced oxidative stress. (A) Images of the colony formation and clonogenic survival assay of A549 LKB1 isogenic pairs treated with indicated dose of IR. Quantification of surviving from clonogenic survival assay for (B) A549, (C) H460 and (D) Calu-6 LKB1 isogenic pairs treated with varying doses of ionizing irradiation. Surviving fraction is calculated as the plating efficiency of treated cells divided by plating efficiency of untreated cells. DER is the dose enhancement ratio at surviving fraction of 0.5. (E) Images of the colony formation and (F) quantification of surviving fraction from clonogenic survival assay for A549 KEAP1 isogenic pairs treated with varying doses of ionizing irradiation. Graphs show means for all biological replicates from 3-6 independent experiments. (G) FACS analysis and bar graph for quantification of intracellular ROS levels were monitored using MitoSOX. (H) FACS analysis and bar graph for quantification of apoptosis assays were monitored using PE-conjugated annexin-V/7-AAD staining in LKB1 and KEAP1 A549 isogenic pairs at 72 h after 8 Gy of IR. All data are presented as mean $\pm$ SD (panels B and F) or SEM; Error bars indicate $\pm$ SD or SEM. A representative experiment from 3 or more experiments is shown.

Figure 4. Loss of LKB1 and KEAP1 promotes radiotherapy resistance in A549 NSCLC xenografts.

In vivo tumor growth and Kaplan-Meier curves of mice bearing (A&B) A549 vector control (N = 8/group); (C&D) LKB1 overexpressed (N = 7-8/group); and (E&F) KEAP1 overexpressed (N = 7/group) -derived xenografts model in nude mice, respectively with or without radiation (5 Gy x1). Tumors were irradiated after reaching 250-350 mm³. Mice were euthanized when tumor volume reached 2,000 mm³. All data are presented as mean $\pm$ SEM; Error bars indicate $\pm$ SEM for each group. Statistical significance. Kaplan-Meier curves show percent of PFS (time to tumor doubling). The *P* values are from log-rank test.

Figure 5. GLS inhibitor radio-sensitizes LKB1 deficient NSCLC cells.

FACS analysis of intracellular ROS and bar graph of quantification of 3 independent experiments for (A) A549 control, (B) A549 LKB1 and (C) A549 KEAP1 cell lines at 72 hours after 1 μ M of CB-839 treatment for 4 h before 8 Gy of IR. FACS analysis and bar graph of quantification from 3 independent experiments of apoptosis assays quantified using PE-conjugated annexin-V/7-AAD staining in (D) A549 control, (E) A549 LKB1, and (F) A549 KEAP1 isogenic pairs at 72 hours with 1 μ M of CB-839 treatment 4 hours before 8 Gy of IR. Quantification of surviving from clonogenic survival assay for (G) LKB1 or (H) KEAP1 A549 isogenic pairs treated with 10nM of CB-839 4 hours before varying doses of radiation. (I) Quantification of surviving from clonogenic survival assay for A549 LKB1 and KEAP1 isogenic pairs treated with 10nM of CB-839 for 4 hours. All data are presented as mean $\pm$ SD (panel F) SEM; Error bars indicate $\pm$ SD or SEM. A representative experiment from 3 or more experiments is shown. Statistical significance ns, not significant).

Table 1.

Clinical characteristics of the GEMINI database of patients with stage III NSCLC treated with definitive radiation according to *STK11/LKB1* mutation status.

Covariate	Levels	<i>STK11/LKB1</i>			p_value
		ALL (N=164)	Wild (N=152)	Mutation (N=12)	
Age (Mean ± Std, Median (Min, Max))		63.2 ± 9.5	63.5 ± 9.5	59.5 ± 8.8	.1050
		64 (28, 86)	64 (28, 86)	58.5 (78, 73)	
Sex	F	84(51.2%)	77(50.7%)	7(58.3%)	.7666
	M	80(48.8%)	75(49.3%)	5(41.7%)	
Chemotherapy	0	4(2.4%)	4(2.6%)	0(0%)	1.000
	1	160(97.6%)	148(97.4%)	12(100%)	
PS	Unknown	11			.6562
	0	55(35.9%)	50(35.2%)	5(45.5%)	
	1	94(61.4%)	88(62%)	6(54.5%)	
	2	4(2.6%)	4(2.8%)	0(0%)	
Smoking	Unknown	1			.1188
	Current	22(13.5%)	18(11.9%)	4(33.3%)	
	Former	112(68.7%)	105(69.5%)	7(58.3%)	
	Never	29(17.8%)	28(18.5%)	1(8.3%)	
PathologicDx_cat	Unknown	1			.0835
	Adenocarcinoma	114(69.9%)	104(68.9%)	10(83.3%)	
	Other	15(9.2%)	13(8.6%)	2(16.7%)	
	SCC	34(20.9%)	34(22.5%)	0(0%)	
Overall Stage Group	IIIA	103(62.8%)	96(63.2%)	7(58.3%)	.7629
	IIIB	61(37.2%)	56(36.8%)	5(41.7%)	
TStage	Unknown	1			.1134
	T0/T1	37(22.7%)	35(23.2%)	2(16.7%)	
	T2	53(32.5%)	52(34.4%)	1(8.3%)	
	T3	43(26.4%)	37(24.5%)	6(50%)	
	T4	30(18.4%)	27(17.9%)	3(25%)	
TStage_cat	Unknown	1			.0362
	T0/T1/T2	90(55.2%)	87(57.6%)	3(25%)	
	T3/T4	73(44.8%)	64(42.4%)	9(75%)	
NStage	Unknown	2			.6031
	N0	6(3.7%)	6(4%)	0(0%)	
	N1	7(4.3%)	6(4%)	1(8.3%)	
	N2	106(65.4%)	99(66%)	7(58.3%)	
	N3	43(26.5%)	39(26%)	4(33.3%)	
Nstage_cat	Unknown	2			1.000
	Negative	6(3.7%)	6(4%)	0(0%)	
	Positive	156(96.3%)	144(96%)	12(100%)	

Abbreviations: F, Female; M, Male; PS, Performance Status; SCC, Squamous cell carcinoma; TStage, Tumor stage; NStage, Nodules stage.

Table 2.

Cumulative incidence rate of LRR, considering DM/Death/New primary as competing risks of patients with stage III NSCLC treated with definitive radiation.

Covariate	Levels	1yr	95% CI LL	95% CI UL	2yr	95% CI LL	95% CI UL	3yr	95% CI LL	95% CI UL	P- value
<i>STK11</i> /LKB1	Wild-Type	0.108	0.065	0.165	0.220	0.157	0.291	0.272	0.202	0.348	0.0108
	Mutation	0.250	0.048	0.531	NA	NA	NA	NA	NA	NA	
Mutation_cat	K	0.083	0.014	0.237	0.130	0.030	0.305	0.185	0.052	0.381	0.2983
	KL	0.143	0.003	0.531	NA	NA	NA	NA	NA	NA	
	KP	0.105	0.016	0.291	0.211	0.060	0.421	0.316	0.118	0.538	
Sex	F	0.136	0.072	0.220	0.265	0.172	0.366	0.306	0.207	0.411	0.3793
	M	0.102	0.047	0.181	0.205	0.123	0.302	0.259	0.166	0.362	
Age_cat	<=70	0.123	0.072	0.188	0.232	0.160	0.311	0.290	0.212	0.374	0.7705
	>70	0.108	0.034	0.233	0.250	0.121	0.402	0.250	0.121	0.402	
PS_cat	0	0.148	0.069	0.257	0.225	0.123	0.346	0.283	0.168	0.410	0.7294
	1, 2	0.093	0.046	0.162	0.232	0.152	0.322	0.279	0.191	0.373	
Smoking_cat	Current/Former	0.108	0.062	0.169	0.229	0.159	0.305	0.280	0.204	0.361	0.7088
	Never	0.172	0.061	0.331	0.276	0.126	0.449	0.310	0.150	0.486	
TStage_cat	<=7	0.114	0.058	0.190	0.195	0.119	0.284	0.245	0.159	0.341	0.4019
	>7	0.127	0.062	0.217	0.276	0.175	0.387	0.321	0.213	0.434	
NStage_cat	Negative	0.000	NaN	NaN	NA	NA	NA	NA	NA	NA	0.9982
	Positive	0.125	0.078	0.183	0.240	0.175	0.311	0.283	0.212	0.357	
PathologicDx_cat	Adenocarcinoma	0.109	0.059	0.175	0.232	0.157	0.315	0.291	0.207	0.380	0.8264
	Other	0.200	0.043	0.438	0.200	0.043	0.438	0.200	0.043	0.438	
	SCC	0.121	0.037	0.259	0.273	0.132	0.434	0.307	0.156	0.472	

Abbreviations: 95% CI, 95% confidence interval; LRR, loco regional recurrence; F, Female; M, Male; PS, Performance Status; TStage, Tumor stage; NStage, Nodules stage; K, *KRAS*-mutant; KL, *KRAS* and *STK11*/LKB1-mutant; KP, *KRAS* and *TP53*-mutant; SCC, Squamous cell carcinoma.

Table 3.

Univariate Cox model for DFS of patients with stage III NSCLC treated with definitive radiation.

Covariate	Level	HR	95% CI		p-Value	Event	Censored	Total
			Low	High				
<i>STK11</i> /LKB1	Mutation vs. Wild-Type	2.530	1.375	4.657	0.0029	138	26	164
Mutation_cat [*]	K vs. KP	0.857	0.441	1.664	0.6478	43	8	51
	KL vs. KP	2.387	0.962	5.923	0.0607			
Age_cat	>70 vs. ≤70	0.890	0.590	1.342	0.5779	138	26	164
Sex	F vs. M	1.019	0.729	1.426	0.9112	138	26	164
PS_cat	0 vs. 1-2	0.871	0.605	1.253	0.4565	128	25	153
Smoking_cat	Current/Former vs. Never	0.971	0.625	1.510	0.8964	137	26	163
PathologicDx_cat	Adenocarcinoma vs. SCC	0.990	0.652	1.502	0.9617	137	26	163
	Other vs. SCC	1.470	0.763	2.834	0.2497			
TStage_cat	≤7 vs. >7	0.818	0.583	1.147	0.2431	137	26	163
NStage_cat	Negative vs. Positive	1.438	0.584	3.537	0.4296	136	26	162

Abbreviations: 95% CI, 95% confidence interval; F, Female; M, Male; K, *KRAS*-mutant; KL, *KRAS* and *STK11*/LKB1-mutant; KP, *KRAS* and *TP53*-mutant; DFS, Disease-free survival; HR, Hazard ratio; PS, Performance Status; TStage, Tumor stage; NStage, Nodules stage; SCC, Squamous cell carcinoma.

* Overall p-value for *KRAS* mutation is 0.0738.

STK11/LKB1 is the single significant factor in DFS.

Table 4.

Univariate Cox model for OS of patients with stage III NSCLC treated with definitive radiation.

Covariate	Level	HR	95% CI		p-Value	Event	Censored	Total
			Low	High				
<i>STK11</i> /LKB1	Mutation vs. Wild-Type	2.198	1.097	4.405	0.0263	96	68	164
Mutation_cat	K vs. KP	1.004	0.446	2.260	0.9924	29	22	51
	KL vs. KP	1.364	0.478	3.894	0.5623			
Age_cat	>70 vs. ≤70	1.808	1.129	2.895	0.0137	96	68	164
Sex	F vs. M	0.940	0.628	1.409	0.7652	96	68	164
PS_cat	0 vs. 1-2	1.022	0.652	1.600	0.9257	90	63	153
Smoking_cat	Current/Former vs. Never	0.991	0.591	1.661	0.9731	96	67	163
PathologicDx_cat	Adenocarcinoma vs. SCC	0.726	0.450	1.171	0.1892	95	68	163
	Other vs. SCC	1.322	0.625	2.796	0.4656			
TStage_cat	≤7 vs. >7	0.974	0.650	1.460	0.8983	96	67	163
NStage_cat	Negative vs. Positive	1.050	0.332	3.325	0.9336	95	67	162

Abbreviations: 95% CI, 95% confidence interval; OS, Overall survival; K, *KRAS*-mutant; KL, *KRAS* and *STK11*/LKB1-mutant; KP, *KRAS* and *TP53*-mutant; HR, Hazard ratio; F, Female; M, Male; PS, Performance Status; TStage, Tumor stage; NStage, Nodules stage; SCC, Squamous cell carcinoma.

Table 5.

Multicovariate Cox Model for OS of patients with stage III NSCLC treated with definitive radiation.

Analysis of Maximum Likelihood Estimates					
Parameter		HR	95% CI		p-value
Age	>70 vs ≤70	1.777	1.109	2.848	0.0169
<i>STK11/LKB1</i>	Mutation vs Wild-type	2.123	1.060	4.252	0.0337

Abbreviations: 95% CI, 95% confidence interval; OS, Overall survival; HR, Hazard ratio.