Molecular Cancer Research

Adapting a Drug Screening Platform to Discover Associations of Molecular Targeted Radiosensitizers with Genomic Biomarkers

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Abstract

Large collections of annotated cancer cell lines are powerful tools for precisely matching targeted drugs with genomic alterations that can be tested as biomarkers in the clinic. Whether these screening platforms, which utilize short-term cell survival to assess drug responses, can be applied to precision radiation medicine is not established. To this end, 32 cancer cell lines were screened using 18 targeted therapeutic agents with known or putative radiosensitizing properties (227 combinations). The cell number remaining after drug exposure with or without radiation was assessed by nonclonogenic assays. We derived short-term radiosensitization factors (SRF_{2Gy}) and calculated clonogenic survival assay–based dose enhancement factors ($DEF_{SF0.1}$). Radiosensitization was characterized by SRF_{2Gy} values of mostly ~1.05 to 1.2 and significantly correlated with drug-induced changes in apoptosis and senescence frequencies. SRF_{2Gy} was significantly correlated with $DEF_{SF0.1}$, with a respective sensitivity and specificity of 91.7% and 81.5% for a 3-day endpoint, and 82.8% and 84.2% for a robotic 5-day assay. *KRAS* mutations (codons 12/13) were found to be a biomarker of radiosensitization by midostaurin in lung cancer, which was pronounced under conditions that enriched for stem cell–like cells. In conclusion, although short-term proliferation/survival assays cannot replace the gold-standard clonogenic survival assay for measuring cellular radiosensitivity, they capture with high accuracy the relative change in radiosensitivity that is caused by a radiosensitzing targeted agent.

Implications: This study supports a paradigm shift regarding the utility of short-term assays for precision radiation medicine, which should facilitate the identification of genomic biomarkers to guide the testing of novel drug/radiation combinations. *Mol Cancer Res; 13(4); 713–20. ©2015 AACR.*

Introduction

Large panels of annotated cancer cell lines provide useful preclinical models for identifying genotype-correlated drug sensitivities that can be clinically tested (1–5). The basic concept underlying the success of these analyses predicts that the cytostatic or cytotoxic effects of drugs in cultured cells translate into tumor regression, a standard criterion of efficacy in patients with metastatic cancer. However, regression is an insufficient surrogate endpoint for the outcome of radiotherapy with curative intent which requires eradication of all tumor cells that could give rise to a local recurrence (6). Traditionally, these have been termed "clonogenic" cells, i.e., cells that have the capacity to produce an expanding family of daughter cells and form colonies following irradiation in an *in vitro* assay or give rise to a recurrent tumor in *in vivo* models. To which extent clonogenic cells may represent cancer stem cells is unclear, though more recently the terms have been used interchangeably (7, 8).

Because chromosomal damage caused by ionizing radiation (IR) may persist over several cell cycles before disrupting a cell's ability to divide infinitely, colony formation or clonogenic survival assays (CSA) have been considered the "gold standard" for assessing the cytotoxic effects of IR in cell culture, supporting the concept that cellular radiosensitivity is a major, though not the only, determinant of *in vivo* radiosensitivity (9–14). In contrast, it is a long-held paradigm that radiosensitivity determined in short-term assays that measure cell proliferation or viability over a few days correlates poorly with radiosensitivity derived from CSA (15, 16).

The importance of preclinical and clinical drug development with IR and its challenges have been highlighted (17–20). Historically, the choice of radiosensitizers has conformed to a "one-size-fits-all" philosophy, but it has become increasingly apparent that radiosensitizing effects may be genotype-dependent, requiring predictive biomarkers for appropriate patient selection (21, 22). To this end, precision radiation medicine may leverage genomic information derived from human cancer cell lines or

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tissue samples. Unfortunately, CSA are not ideal for the large-scale and high-throughput cell line screens that would be needed to identify tumor genotypes that correlate with sensitivity to IR/drug combinations owing to the often poor colony-forming ability of human cancer cell lines and the time and resources it takes to conduct these assays. This is an important barrier to preclinical testing and clinical translation of novel IR/drug regimens.

We recently observed that the radiosensitizing effects of EGFR inhibitors seen in a short-term viability assay correlated well with radiosensitization in a CSA because the premature senescence response underlying radiosensitization led to a proliferative delay that was captured in the 3-day assay (23). We, therefore, hypothesized that short-term assays can provide a measure of the change in cellular radiosensitivity that is caused by a targeted drug provided the drug alters the mode of cell inactivation observed within a few days following irradiation, such as senescence, apoptosis, or autophagy. Furthermore, we reasoned that robotic screening platforms can be adapted to capture the ultimately therapeutically significant but small magnitude effects of radiosensitizing drugs (~10% reduction in cell number) which stand in contrast with the typically large effects of targeted drugs alone in susceptible cell lines (>50% reduction; refs. 23–25).

Materials and Methods

Cell lines

Annotated cell lines were selected from previously published panels (1, 23–25). The identity of the cell lines had been tested as described (23), and additional authentication was performed by Bio-Synthesis, Inc. No cell line tested positive for mycoplasma (MycoAlert; Lonza). For three-dimensional (3D) culture of tumor spheres, 5,000 cells per well were grown in low-binding 96-well plates (Thermo; 145399) using serum-free medium composed of DMEM (Sigma-Aldrich), basic fibroblast and epidermal growth factor (20 ng/mL each; Sigma-Aldrich), and B27 supplement (Life Technologies), followed by drug/IR treatments 3 days later.

Treatments

X-ray treatments were performed as described (23). Drugs were dissolved in DMSO (Sigma-Aldrich), except chloroquine, which was dissolved in deionized water. Drugs were aliquoted and stored according to the manufacturers' guidelines. Drugs were added to cells 1 hour before irradiation at appropriate concentrations (Supplementary Table S1).

Cell survival assays

Clonogenic cell survival was measured by seeding cells for colony formation at appropriate densities 16 to 18 hours before 2 Gy irradiation ± drug preincubation as described (23, 25). Drugs were not washed out following irradiation except for NU7026 and olaparib after 24 hours. The syto60 assay has been described (23–25). The CellTiter-Glo (CTG) luminescence (Promega) and MTT metabolic assays (Cayman Chemical) were performed following the manufacturer's protocols. To adapt robotic screening (1), 96-well clear bottom black plates (Corning) with optimized cell density for each cell line (i.e., 70%–80% confluence by end of the assay for control samples) were prepared. Cells were drugged by the liquid handling robot (Zephyr; Caliper Life Sciences) 1-hour preirradiation. CTG reagents were applied to cells 5 days later (EL406; BioTek Instruments). Signals were read by the MultiLabel reader, 2140 Envision (Perkin Elmer).

Apoptosis and senescence assays

Seventy-two hours after irradiation, cells and media were collected, centrifuged, and resuspended in Annexin binding buffer with cell density adjusted to approximately 10^6/mL. Cells were stained with propidium iodide (Sigma-Aldrich) and Annexin V–Cy5 following the manufacturer's protocol (BioVision), and then analyzed by an LSRII flow cytometer (BD Biosciences). Senescence-associated β-galactosidase staining was performed using a commercial kit (Cell Signaling; #9860) as described (23).

Immunofluorescence microscopy

Staining and visualization of γ-H2AX and 53BP1 foci were performed as previously described (23, 25).

Western blotting

Whole cell lysates were prepared using standard methods. Specific antibodies against phospho-PKC (pan) (Cell Signaling; #9371) and total PKCα [Y124] (Abcam; ab32376), and horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology) were used. Protein bands were visualized with enhanced chemiluminescence (Invitrogen) followed by autoradiography.

RNA interference

PKCα siRNA transfections were carried out as described (25).

Flow cytometry

Cells were labeled with CD133/1 (AC133)–PE antibody (Miltenyi Biotec), and high and low CD133-expressing cells were subjected to sterile sorting by flow cytometry.

Statistical analysis

All data were analyzed by GraphPad Prism 6. Clonogenic survival data were fitted by the linear-quadratic (LQ) formula. Statistical comparisons were carried out with the *F* test. Unless noted, all statistical tests were two-sided. Receiver operating characteristics (ROC) curves were applied to evaluate the performance of the short-term screening platforms in comparison with the CSA to determine an appropriate cutoff for the testing assay.

Results

Nonclonogenic screening platform

To determine the effects of irradiation ± drug treatment on short-term survival of cancer cells, we initially employed a previously published assay that relies on fixation of persistent cells followed by incubation with a nucleic acids stain (syto60) for quantification (refs. 23, 24; Supplementary Fig. S1A–S1E), in addition to other common short-term assays (CTG and MTT; Supplementary Fig. S1F–S1I). We arbitrarily selected a pilot panel of 32 cell lines derived from lung, colorectal, genitourinary, and head and neck cancers as well as 18 molecular targeted drugs with known or putative radiosensitizing properties (1). Drug concentrations were selected to be minimally toxic for drug alone treatments, known to inhibit the target, and achievable in patients. Drugs were added to plates 1 hour before mock treatment or irradiation with a clinically relevant dose of 2 Gy followed by incubation for ≥3 days depending on the particular experiment (Fig. 1A). In total, we assayed 227 cell line–drug combinations (Supplementary Table S1A and S1B). The effect of combined drug/IR relative to the effect of IR alone, and corrected for drug

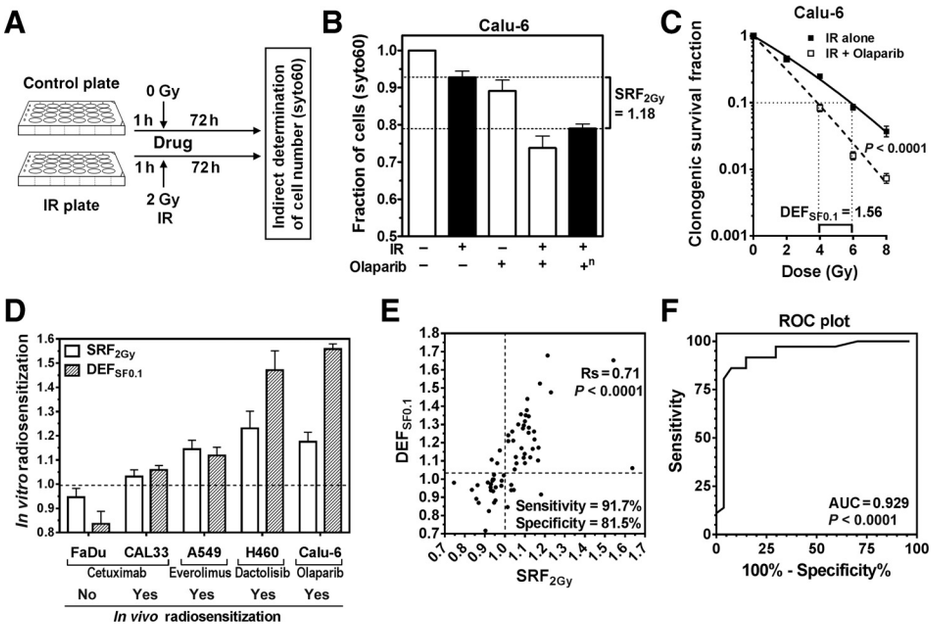

Figure 1.

Correlation of radiosensitization in short-term syto60 and clonogenic assays. A, illustration of pilot setup using a 24-well format and syto60 staining. h, hours. B, example of short-term radiosensitization using the PARP inhibitor olaparib in Calu-6 lung cancer cells. SRF_{2Gy}, short-term radiosensitization factor for 2 Gy (for definition, see Supplementary Fig. S1J). +, treatment with 2 Gy IR or/and 1 μmol/L olaparib; –, no treatment; *n*, effect of combined IR and olaparib normalized for the effect of olaparib alone. C, example of radiosensitizing drug effect using clonogenic survival as endpoint. $DEF_{SF0.1}$, which is a standard descriptor of radiosensitization (14), represents the ratio of radiation doses required to achieve 0.1 clonogenic survival when given without and with drug. The drug + IR curve is corrected for the effect of drug alone. Statistical comparison by the *F* test. D, illustration of the association of SRF_{2Gy} with $DEF_{SF0.1}$ values and previously reported radiosensitization of xenografts for various cell lines and targeted drugs (see text). E, correlation of SRF_{2Gy} with $DEF_{SF0.1}$ for 25 cancer cell lines treated with up to 8 agents for a total of 63 comparisons. $DEF_{SF0.1}$ values were derived from full clonogenic survival curves (Supplementary Table S1A). For sensitivity and specificity calculations, cutoffs of $\geq$1.01 for SRF_{2Gy} and $\geq$1.04 for $DEF_{SF0.1}$ were used to define a positive effect. Statistical comparison by the Spearman rank correlation. Rs, Spearman rank coefficient. F, ROC curve using the data shown in E. AUC, area under the curve.

alone effect, was expressed as SRF_{2Gy} (short-term radiosensitization factor at 2 Gy; Supplementary Fig. S1B and S1J).

Correlation of radiosensitization in nonclonogenic and clonogenic survival assays

To correlate radiosensitization in the short-term syto60 assay (SRF_{2Gy}) with radiosensitization using the CSA, standard dose enhancement factors at 0.1 clonogenic survival fraction ($DEF_{SF0.1}$; ref. 14) were calculated (illustrated in Fig. 1C). Initial experiments using selected targeted drugs and cell lines suggested that radiosensitization described by SRF_{2Gy} not only predicted drug effect in the CSA, but also correlated with the known ability of these drugs to enhance the effects of IR *in vivo* (Fig. 1D; refs. 26–29).

Next, we generated 63 comparisons of syto60-based SRF_{2Gy} values and CSA-derived $DEF_{SF0.1}$ values based on 25 cancer cell lines treated with up to 8 drugs (Fig. 1E; Supplementary Table S1A). There was a highly statistically significant correlation between SRF_{2Gy} and $DEF_{SF0.1}$ values ($P < 0.0001$). Similarly, short-term and clonogenic SRF_{2Gy} values were significantly correlated ($P < 0.0001$; Supplementary Fig. S2A). An ROC plot confirmed the high accuracy of SRF_{2Gy} values to predict radiosensitization ($P < 0.0001$; Fig. 1F). Notably, even small SRF_{2Gy} values of 1.05 or less were often associated with radiosensitization in the CSA (Fig. 1E) so that we selected a cutoff of $\geq$1.01. For $DEF_{SF0.1}$, we chose a cutoff of $\geq$1.04 due to data indicating that even DEF values this small could be clinically significant (Fig. 1D; Supplementary Fig. S2B). With these cutoffs, the overall sensitivity and specificity with regard to radiosensitization in the CSA were 91.7% and 81.5%, respectively.

We conclude that a short-term assay can capture the relative change in radiosensitivity caused by a radiosensitizing agent. Thus, specifically for radiosensitization, short-term endpoints may be an appropriate surrogate of CSA. However, our data do not suggest that short-term assays should be generally substituted for CSA. In fact, we did not find any correlation between cellular radiosensitivity measured with the short-term assay and radiosensitivity determined using the CSA (Supplementary Fig. S2C), which is consistent with historical data (15, 16).

Drug-induced changes in apoptosis and senescence correlate with radiosensitization

Notably, the SRF_{2Gy} values that correlated with radiosensitization in the CSA were generally small, i.e., on average 1.12 (SD, ± 0.13; Fig. 1E, and further illustrated in Supplementary Fig. S2D). To increase our confidence that these small values represent true effects, we tested an alternate 2 × 2 Gy irradiation schedule because during a fractionated course of radiotherapy in the clinic, the cytotoxic effect of a single dose is repeated. This schedule produced statistically significant increases in SRF_{2Gy} for several cell–drug combinations (Fig. 2A). In addition, because IR-induced lethal chromosomal aberrations may inactivate cells only after a few cell divisions, we extended the incubation period from 3 to 6 days, which also yielded an often pronounced increase in SRF_{2Gy} (Fig. 2A and Supplementary Fig. S2E).

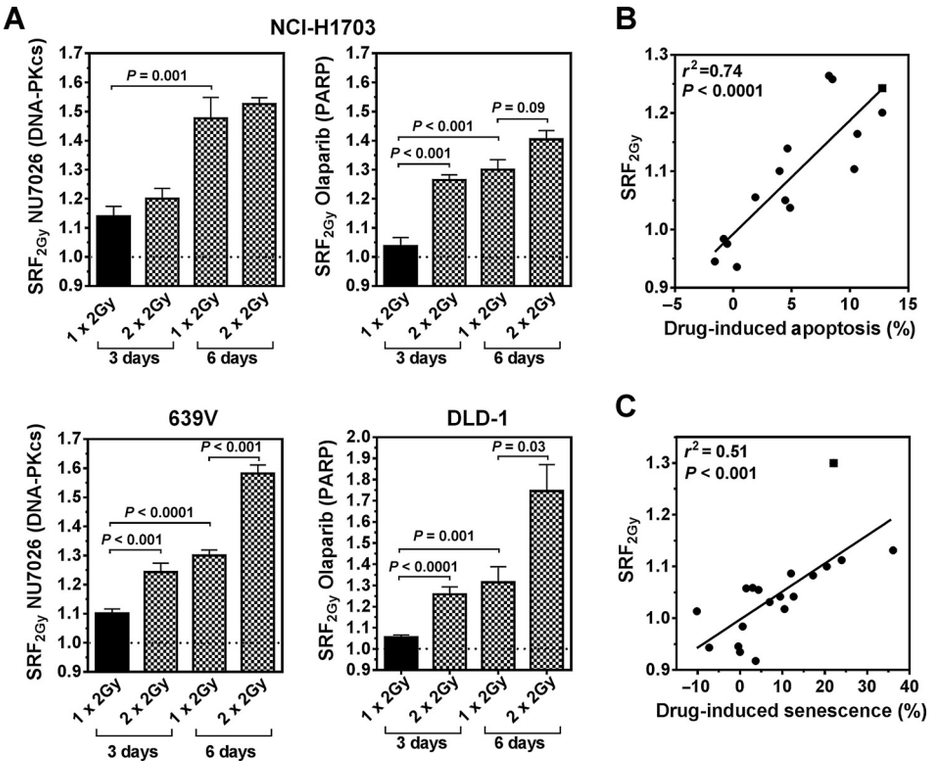

Figure 2.
Factors that enhance short-term radiosensitization and correlation with apoptosis and premature senescence frequencies. A, examples of enhanced SRF_{2Gy} when IR is repeated (2 Gy × 2, 24 hours apart) or when incubation times are extended to 6 days (incubation times counted from day of (first) irradiation). Data points shown represent mean (± SE) based on at least three biologic repeat experiments. Statistical comparisons by unpaired *t* test, two-sided. B, correlation of SRF_{2Gy} values with relative change in the percentage of Annexin V–positive cells upon adding drug to IR, normalized for drug alone effect. Data points represent differences between drug + 2 Gy versus 2 Gy alone effects in several cell lines, except for square symbol, which indicates a 2 × 2 Gy treatment. Solid line, result of linear regression analysis. C, analogous to B, correlation of SRF_{2Gy} with relative change of SA-β-gal–positive cells scored 3 days after irradiation, except for square symbol, which represents a 6-day experiment.

Next, we investigated the cellular events underlying the observed radiosensitization by different drugs. A strong correlation between drug-induced apoptosis and SRF_{2Gy} was found for several cell line–drug combinations (Fig. 2B; Supplementary Fig. S3A–S3E). This is particularly well illustrated in NCI-H1703 cells, which are senescence-resistant due to nonfunctional p53/p16 (Supplementary Fig. S3A–S3C). Drug-induced premature senescence could also be observed, as shown in Supplementary Fig. S4, and correlated well with radiosensitization (Fig. 2C). Together, the data in Fig. 2 suggest that the observed SRF_{2Gy} values (Fig. 1E) represent not only true effects that are based on drug-induced changes in apoptosis or senescence responses but also in many cases can be augmented by fractionation and/or prolongation of incubation times.

Implementing a robotic high-throughput platform for personalized radiation medicine

To adapt our approach for robotic high-throughput screening (1), we confirmed that the observed radiosensitizing effects were not specific to the syto60 assay and could be detected with the commonly used MTT and CTG assays ($P < 0.0001$; Fig. 3A). Comparative analysis using a 96-well plate format indicated that the CTG assay was the most sensitive and robust of the three assays, and was thus selected for robotic platform testing (Fig. 3B and Supplementary Fig. S1G–S1I). Ten cancer cell lines and 16 targeted drugs were chosen (Supplementary Table S1B). Clonogenic survival data were available for 48 cell line–drug combinations, and indicated a high accuracy of the CTG assay in terms of predicting radiosensitization, with a sensitivity of 82.8% and specificity 84.2% (Fig. 3C and D). A higher cutoff for SRF_{2Gy} of $\geq$1.04 was chosen compared with the syto60 assay, given the tendency of the CTG assay to produce generally slightly higher SRF_{2Gy} values.

Genomic biomarkers of radiosensitization

Next, we focused on a subset of lung cancer cell lines to determine if our screening platform can detect genetically defined mechanisms of radiosensitization. For this, we arbitrarily selected the mTOR inhibitor everolimus, a negative regulator of DNA damage–mediated autophagy, and the multikinase inhibitor midostaurin (30–33). For everolimus, radiosensitization was observed almost exclusively in cell lines with wild-type *TP53* ($P = 0.001$; Fig. 4A), and this was confirmed in an isogenic cell pair (Supplementary Fig. S5A and S5B). Consistent with a promoting role of p53 in autophagy induction and premature senescence (23, 34, 35), we observed everolimus-induced autophagy and senescence only in irradiated *TP53* wild-type but not mutated cells (Supplementary Fig. S5C and S5D). Of the top 5 cell lines radiosensitized by midostaurin (SRF_{2Gy} of 1.02–1.13), 4 harbored *KRAS* mutations in codons 12 and 13 (Fig. 4B). In contrast, cells with wild-type *KRAS* or mutations in codons 61 did not show radiosensitization ($P = 0.01$). *KRAS* codon 12/13 mutation-dependent radiosensitization was confirmed in isogenic cell pairs and the CSA (Fig. 5A–C). Midostaurin also increased the number of residual IR-induced DNA double-strand breaks and caused apoptosis and senescence in irradiated *KRAS*-mutant cells (Supplementary Fig. S6A–S6C), in line with the correlations shown in Fig. 2B and C.

Interestingly, we recently found that PKCα, a known target of midostaurin, contributes to the radioresistance of *KRAS*-mutant cells (25). We, therefore, compared the radiosensitizing effect of a specific PKCα small molecule inhibitor with the effect of

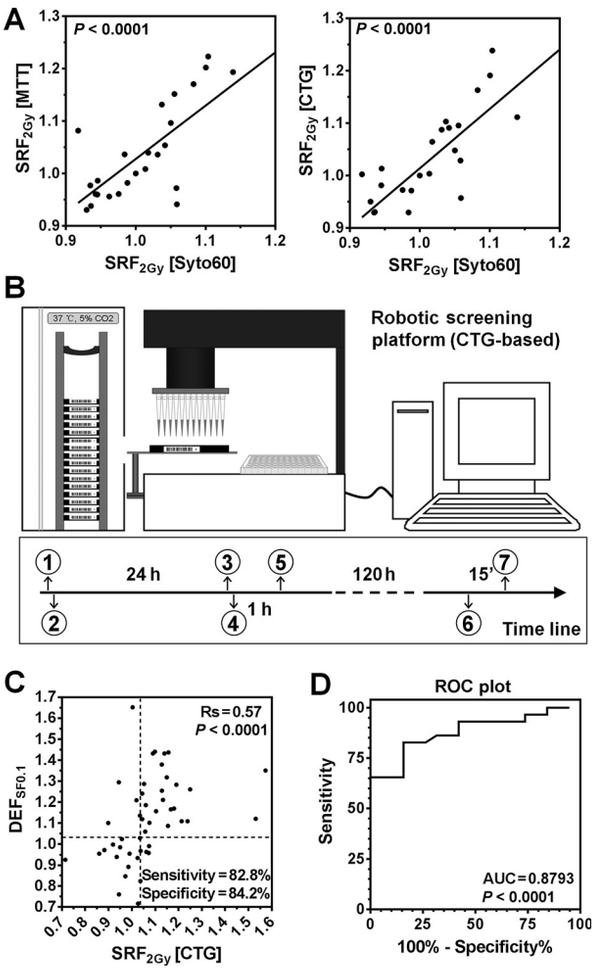

Figure 3.
Establishing a robotic cell line screening platform. A, comparison of the syto60 assay with the MTT or CTG assay. Solid lines and *P* values represent results of linear regression analysis. Each data point is based on three biologic repeats. B, illustration of the robot-assisted screening process. 1, prepare duplicate plates (mock treatment, IR 2 Gy); 2, seed one cell line per plate; 3, prepare master drug plate; 4, load plates, add drugs via robot, and incubate; 5, treat plates and return to incubator; 6, add CTG agents; 7, read plates. C, correlation of SRF_{2Gy} values obtained with the 5-day robotic CTG assay and $DEF_{SF0.1}$ values analogous to Fig. 1E. For sensitivity and specificity calculations, cutoffs of $\geq$1.04 for SRF_{2Gy} and $\geq$1.04 for $DEF_{SF0.1}$ were chosen to define a positive effect. Data points represent 48 comparisons based on 9 cell lines and 12 targeted agents. D, ROC plot analogous to Fig. 1F.

midostaurin and observed comparable results (Fig. 5D). Depletion of PKCα abrogated the radiosensitizing effect of midostaurin (Fig. 5; Supplementary Fig. S6D). As PKCα was recently implicated in maintaining breast cancer stem cells (36), we asked whether midostaurin's effect was more pronounced in a subpopulation of lung cancer cells. Strikingly, midostaurin poorly radiosensitized cells with low expression of the stem cell marker CD133, whereas a relatively large SRF_{2Gy} of 1.43 was observed in a subpopulation of high CD133 expressors (Fig. 5F and Supplementary Fig. S6E). Thus, a relatively small SRF_{2Gy} seen in an unselected cell population, such as ~1.1, for midostaurin may be driven by the sensitivity of a stem cell–like subpopulation. Tumor spheres are thought to contain a higher fraction of stem cells compared with monolayer cultures (36). Again, the radiosensitizing effect of midostaurin was evident and enhanced in *KRAS*-mutant tumor spheres, i.e., $SRF_{2Gy} \sim 1.4$ (Fig. 5G and Supplementary Fig. S6F).

Discussion

Clonogenic survival assays have been considered the gold standard for assessing the cell-inactivating effects of IR *in vitro* (37–39). Even though plate formats have been tested (38, 40, 41),

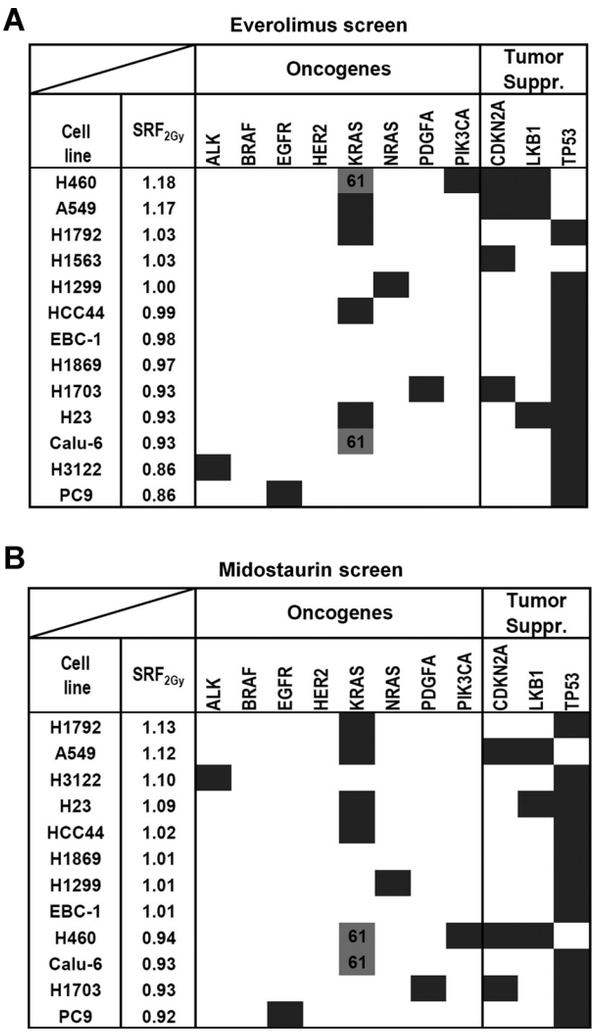

Figure 4.
Identification of genomic biomarkers of radiosensitization by the mTOR inhibitor everolimus and the multikinase inhibitor midostaurin. A, results of IR/drug screen of 13 lung cancer cell lines with everolimus (20 nmol/L; Supplementary Table S1A). Cell lines are ranked by average SRF_{2Gy} value. Dark fields indicate known mutations or other genomic alterations in common oncogenes and tumor suppressors (suppr.). *KRAS* codon 61 mutations (gray fill-in) are distinguished from codon 12/13 mutations (dark). SRF_{2Gy} values are statistically significantly higher in TP53 wild-type than in mutated cell lines ($P = 0.001$; *t*-test). B, analogous results for midostaurin (100 nmol/L). SRF_{2Gy} values are statistically significantly higher in cell lines with *KRAS* codon 12/13 mutations than in all other cell lines ($P = 0.01$) and higher than in wild-type cell lines ($P = 0.04$).

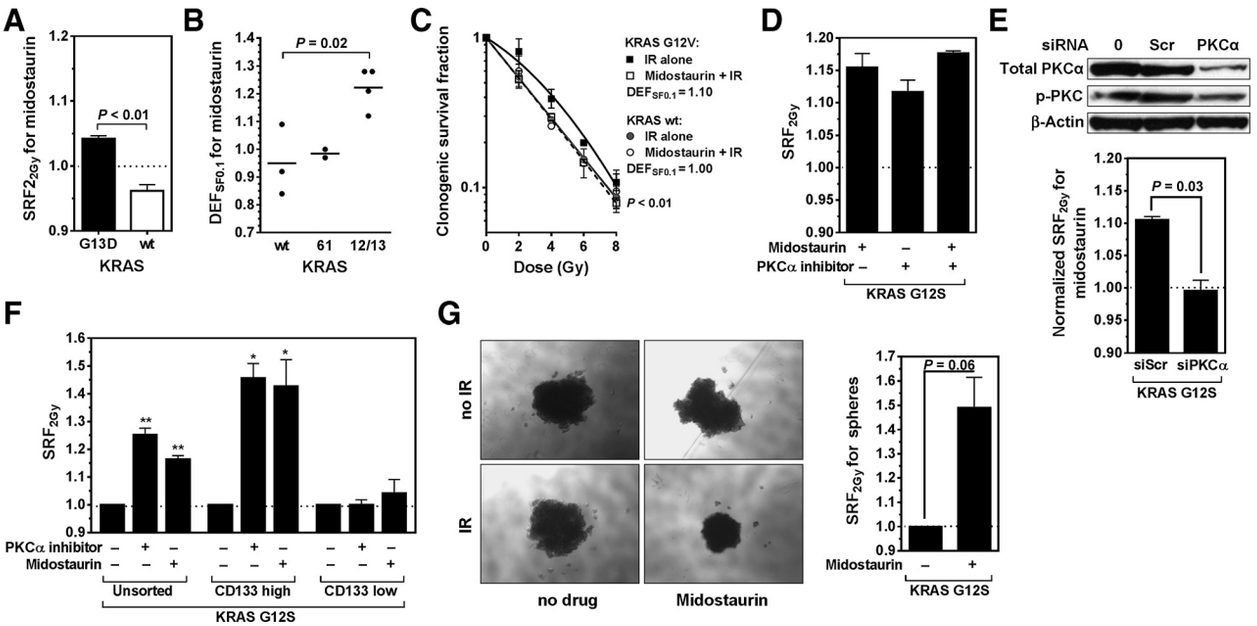

Figure 5.

Follow-up analysis on the radiosensitizing effects of midostaurin. A, SRF_{2Gy} values for isogenic DLD-1 cells harboring a mutant *KRAS* or a deleted allele. B, $DEF_{SF0.1}$ values derived from clonogenic survival curves according to *KRAS* status of nonisogenic lung cancer cell lines. Statistical comparison with *t* test. C, clonogenic survival of isogenic *KRAS* wild-type NCI-H1703 cells with or without stable expression of a mutant *KRAS* transgene. Data were fitted using the LQ formula, and statistical comparison was performed with the *F* test. D, SRF_{2Gy} values for *KRAS*-mutated A549 cells treated with midostaurin or the PKCα-specific Ro-32-0432 inhibitor (100 nmol/L). E, effect of PKCα depletion: top, Western blot of A549 cells transfected with no reagents (0), a scrambled control (scr), or siRNA against PKCα. p-PKC indicates a pan-antibody against phospho-PKC isoforms. Bottom, SRF_{2Gy} values for midostaurin with or without PKCα depletion. Statistical comparison with *t* test. F, SRF_{2Gy} values in A549 populations based on treatment with midostaurin or Ro-32-0432 following no sorting, sorting for high CD133 expressors, and sorting for low CD133 expressors. Statistical comparisons to IR alone with one-sample *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$. G, Left: representative images of A549 spheres following treatments as indicated. Right, quantification of results using the CTG assay. Statistical comparison with *t* test. All data points shown represent mean ± SE based on at least three biologic repeat experiments.

CSA are not ideal for the high-throughput screens that are needed to match genomic tumor profiles with IR/drug sensitivities owing to the frequently poor colony-forming ability of human cancer cell lines and the time it takes to conduct these assays. Short-term cell viability/survival assays, on the other hand, are generally not considered to provide appropriate surrogate endpoints of clonogenic survival (15, 16, 41). In individual cell lines, some short-term assays, such as the MTT assay, can capture radiosensitizing effects and correlate with CSA (42–44). However, to our knowledge, the utility of short-term assays as a surrogate for CSA for screening any larger number of cancer cell lines has never been validated.

Here, we establish a robust correlation between short-term and clonogenic radiosensitization for a variety of cell lines, drugs, and assay conditions (Figs. 1E and 3C). Short-term radiosensitization was measured for 2 Gy IR single doses which are clinically relevant and largely avoid cell cycle delay problems that can be caused by higher IR doses. The cumulative data suggest that changes in senescence, apoptosis, or/and autophagy caused by the radiosensitizing drug compared with cells treated with IR alone consistently affect the number of persisting cells that are measured by the short-term assays (Fig. 2 and Supplementary Figs. S3 and S4). Because IR-induced lethal chromosomal aberrations typically abrogate cell proliferation after a few division cycles, for example, by causing mitotic catastrophe, apoptosis, or senescence (45), we suggest that assessing cell numbers at approximately 5 to 6 days after irradiation is the best compromise between giving enough time for drug effects to manifest (at least 2–3 days) and avoiding the lengthy incubation needed for selecting out the surviving cells as colonies (2–3 weeks). Our findings provide an important proof of principle regarding the close correlation between short-term and clonogenic assay results when assessing radiosensitizing effects. However, more data will be needed before we can establish robust cutoffs for predicting DEF values (Figs. 1E and 3C).

In contrast with studies on the antiproliferative effects of anticancer drugs alone, which are typically pronounced in susceptible cell lines (24), the dynamic range of radiosensitizing effects when combining drugs with 2 Gy IR is much smaller, though surprisingly robust. For example, in the 3-day syto60 assay, the average SRF_{2Gy} was only approximately 1.1 (Fig. 1E). An SRF_{2Gy} as small as 1.03 for the combination of the EGFR-directed monoclonal antibody cetuximab and IR in the head and neck cancer cell line CAL33 appears to be clinically meaningful given the observed impact *in vivo* (Fig. 1D and Supplementary Fig. S2B; ref. 26). That such small SRF_{2Gy} values capture early cellular events corresponding to real radiosensitization that should translate into larger effects with prolonged radiation courses was further highlighted by several lines of experimentation which demonstrated an increase in SRF_{2Gy} when (i) incubation time was prolonged (to allow for additional senescence and apoptosis events to occur; Fig. 2A and Supplementary Fig. S2E); (ii) repeat 2 Gy irradiation was performed (Fig. 2A and Supplementary Fig. S3B and S3E); and (iii) drug effect was measured under stem

cell–enriched culture conditions (Fig. 5F and G and Supplementary Fig. S6F).

Our initial screens successfully established a genomic biomarker, *KRAS* mutation, for one of the targeted drugs, midostaurin (Fig. 4B; ref. 5). Cells with codon 12/13 mutations were radiosensitized, whereas those with codon 61 mutations were not, suggesting functional heterogeneity associated with different *KRAS* mutations, although we did not pursue this further. Even though midostaurin is a "dirty" tyrosine kinase inhibitor with multiple targets (32, 33), our findings suggest that PKCα is a critical target for radiosensitization of *KRAS*-mutant cells (Fig. 5D and E; Supplementary Fig. S6D; ref. 25). A phase I trial of midostaurin with radiation in rectal cancer is ongoing at our institution (NCT01282502, www.clinicaltrials.gov). The data highlight the potential clinical significance of this type of screening.

As *KRAS* mutations are present in approximately 30% of non–small cell lung carcinoma (46), a relatively small cell line panel was sufficient to detect a potential association with drug effect (Fig. 4B). However, one can envision that drug/IR combinations exist that track with more uncommon genomic alterations, e.g., present in <5% to 10% of tumors. To detect those associations, panels of approximately 50 to 100 cell lines will be needed. This represents a very different approach from traditional investigations of IR/drug combinations which have utilized only small numbers of *in vitro* cell lines for a given cancer type (3, 17), consistent with the traditional "one-size-fits-all" philosophy of combining IR with drugs in patients.

We believe that genomic biomarker discovery using established cancer cell lines has validity given the observed genotype and phenotype similarities with human cancers, though this is not undisputed (47, 48). It is also clear that *in vitro* radiosensitization may not readily translate into *in vivo* effects, and therefore a path to *in vivo* validation of radiosensitizing effects remains a critical part of any preclinical investigation strategy (39, 49, 50).

In conclusion, although short-term assays cannot supplant the gold-standard CSA for measuring absolute radiosensitivity, screening platforms such as ours can capture with high accuracy the relative change in radiosensitivity that is caused by a targeted drug in an individual cell line. Genomic biomarkers identified through this type of screen may guide the identification of patients who would benefit from novel drug/IR combinations. We suggest that our data support a paradigm change regarding the utility of nonclonogenic survival assays in precision radiation medicine.

Disclosure of Potential Conflicts of Interest

T.S. Hong reports receiving a commercial research grant from Novartis and is a consultant/advisory board member for Eisai. J. Settleman is senior director at Genentech. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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