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Detection of Impaired Homologous Recombination Repair in NSCLC Cells and Tissues

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ORIGINAL ARTICLE

Detection of Impaired Homologous Recombination Repair in NSCLC Cells and Tissues

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Introduction: Homologous recombination repair (HRR) is a critical pathway for the repair of DNA damage caused by cisplatin or poly-ADP ribose polymerase (PARP) inhibitors. HRR may be impaired by multiple mechanisms in cancer, which complicates assessing the functional HRR status in cells. Here, we monitored the ability of non–small-cell lung cancer (NSCLC) cells to form subnuclear foci of DNA repair proteins as a surrogate of HRR proficiency.

Methods: We assessed clonogenic survival of 16 NSCLC cell lines in response to cisplatin, mitomycin C (MMC), and the PARP inhibitor olaparib. Thirteen tumor explants from patients with NSCLC were subjected to cisplatin ex vivo. Cells were assayed for foci of repair-associated proteins such as BRCA1, FANCD2, RAD51, and γ-H2AX.

Results: Four cell lines (25%) showed an impaired RAD51 foci-forming ability in response to cisplatin. Impaired foci formation correlated with cellular sensitivity to cisplatin, MMC and olaparib. Foci responses complemented or superseded genomic information suggesting alterations in the ATM/ATR and FA/BRCA pathways. Because baseline foci in untreated cells did not predict drug sensitivity, we adapted an ex vivo biomarker assay to monitor damage-induced RAD51 foci in NSCLC explants from patients. Ex vivo cisplatin treatment of explants identified two tumors (15%) exhibiting compromised RAD51 foci induction.

Conclusions: A fraction of NSCLC harbors HRR defects that may sensitize the affected tumors to DNA-damaging agents including PARP inhibitors. We propose that foci-based functional biomarker assays represent a powerful tool for prospective determination of treatment sensitivity, but will require ex vivo techniques for induction of DNA damage to unmask the underlying HRR defect.

Key Words: Lung cancer, Homologous recombination, RAD51, Biomarker.

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In patients with advanced or metastatic non–small-cell lung cancer (NSCLC) treated with platinum-based chemotherapy regimens, disease response rates are only in the range of 15 to 35%. Although overexpression of the nucleotide excision repair protein ERCC1 or presence of mutant K-RAS in NSCLC have been associated with resistance to platinum-based chemotherapy,[1] there is a paucity of biomarkers to predict which tumors are likely to be sensitive to platinum or targeted agents such as poly-ADP ribose polymerase (PARP) inhibitors.

Homologous recombination repair (HRR) is a pathway critical for several cellular processes including the error-free repair of DNA double-strand breaks (DSB) and the recovery of stalled DNA replication forks.[2] Deregulated HRR results in genomic instability that may cause or contribute to carcinogenesis. Importantly, such HRR-defective tumors may be more sensitive to DNA-damaging chemotherapeutics such as cisplatin or PARP inhibitors. HRR may be altered by genetic, epigenetic, or other mechanisms, making it challenging to assess the functional HRR status in a given tumor. In addition, even though altered expression of genes involved in HRR, such as *BRCA1* or *FANCD2*, has been described in NSCLC, it is unknown whether this is associated with a functionally relevant HRR defect.[3–5] Interestingly, 7% of lung adenocarcinomas harbor mutations in the DNA damage response ATM kinase, which is involved in HRR and

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multiple other DNA repair and checkpoint functions,[6] but the functional consequences of this alteration remain to be established.

Significantly, the activity of HRR and other DNA damage response/repair pathways is less dependent on protein expression levels than on the proper localization of these proteins to sites of damaged DNA, which ensures spatio-temporal coordination and execution of repair.[2] Accordingly, in response to DNA damage, several of these proteins form microscopically visible foci that accumulate in the nuclei of cells. In contrast, in undamaged cells, the frequency of these subnuclear foci is low. Thus, the ability of cells to form repair protein foci may be regarded as a functional biomarker of the integrity of the HRR network and associated sensitivity to cisplatin and other anticancer agents. The advantage of using these foci as biomarkers is that they can detect repair defects due to various mechanisms such as epigenetic events or gene mutations. Moreover, they potentially provide a global measurement of network function without needing to know the identities of all the components.

Here, we sought to discover functionally relevant HRR defects by interrogating a panel of NSCLC cell lines and tissues for their ability to form DNA damage-induced foci of RAD51 recombinase, which is the central effector of HRR in cells.[2]

PATIENTS AND METHODS

Cell Lines

Cell lines were selected from a published panel located in the Center for Molecular Therapeutics at Massachusetts General Hospital, except for A549, Calu-6, and NCI-H2087, which were purchased directly from American Type Culture Collection (Manassas, VA). Cell lines were obtained during 2008–2010 and cultures passaged for less than 3 months after thawing a given frozen vial. In the Center for Molecular Therapeutics, the identity of each of the cell lines in the panel was tested using a set of 16 short tandem repeats (AmpFLSTR Identifier KIT; ABI). In addition, single-nucleotide polymorphism profiles based on a panel of 63 single-nucleotide polymorphisms assayed using the Sequenom Genetic Analyzer (Sequenom, San Diego, CA) was used for in-house identity checking whenever a cell line was propagated and confirmed uniqueness of cell lines for the ones without available short tandem repeats. A549 cells were maintained in Dulbecco's modified Eagle medium, Calu-6 in α-MEM, ABC1, and NCI-H2126 in DMEM/F12, and HCC44, LU99B, NCI-H1299, NCI-H1563, NCI-H1703, NCI-H1792, NCI-H1915, NCI-H2087, and NCI-H23 in NCI-H3122 in RPMI1640 (from Sigma-Aldrich, St. Louis, MO). SV40-transformed human fibroblasts, ATM-mutant AT5BIVA (GM5849A) and ATM wild-type NF (GM00637F), were provided by Simon Powell. Cells were grown in Dulbecco's modified Eagle medium. Maintenance of PD20 with or without expression of wild-type FANCD2 has been described.[7] All cell lines were maintained in a humidified incubator at 37°C and 5% CO_2. All media was supplemented with 10% bovine growth serum (HyClone), 20 mM HEPES, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml stretopmycin (all Sigma-Aldrich). No cell line was ever treated for mycoplasma and all lines were tested mycoplasma-free before the experiments (MycoAlert; Lonza, Basel, Switzerland).

Tumor Tissues

Tumor tissues were obtained from previously untreated patients undergoing surgical resection for clinical stage I NSCLC under a protocol approved by the Boston University Medical School and Partners Institutional Review Boards. Tumor samples were processed for ex vivo foci analysis adapting a previously published protocol for breast cancer.[8] Under this protocol, tumor tissues not needed for pathological diagnosis were placed into RPMI medium typically within 30 minutes of surgical resection and arrived in the laboratory 30 to 60 minutes later. Specimens were evenly divided into samples of less than 5 mm size depending on the amount of tissue available. Samples were mock treated or exposed to 10 Gy radiation, followed by 5 hours incubation at 37°C and 5% carbon dioxide, and snap freezing, as described.[8] In addition to the published protocol, one to two tumor samples were also exposed to cisplatin at 8 μM for 5 hours.

Treatment and Clonogenic Survival Assay

Treatments with cisplatin, mitomycin C, or ionizing radiation were carried out as described.[7,9,10] Olaparib (LC Laboratories, Woburn, MA and AstraZeneca, Wilmington, DE) was dissolved in DMSO for a 20 mM stock solution and stored in aliquots at −20°C. Clonogenic cell survival was assessed as described.[10]

Immunofluorescence Microscopy

Exponentially growing cells were plated into eight-well chamber slides and treated with 8 μM cisplatin as described previously.[10] Visualization of RAD51, FANCD2, and γ-H2AX foci in cell lines was performed as described.[10,11] For BRCA1, cells were incubated with an anti-BRCA1 mouse monoclonal antibody (OP92, Calbiochem) at a 1:200 dilution. For RAD51 and proliferating cell nuclear antigen (PCNA) costaining, PD20 cells were permeabilized first with 0.5% Triton X, 20 mM HEPES, 50 mM NaCl, 3 mM KCl, and 300 mM sucrose on ice for 5 minutes, followed by fixation with 2% paraformaldehyde at room temperature for 20 minutes and 100% methanol at −20°C for 10 minutes. Cells were exposed to primary antibodies against PCNA (rabbit polyclonal ab2426; Abcam, Boston MA) and RAD51 (mouse monoclonal ab213, Abcam) both at 1:200 dilution in 2% bovine serum albumin, 0.1% Triton X-100, followed by incubation with secondary antibodies (Alexa Fluor-488 goat antimouse, Invitrogen A11029, and Alexa Fluor-568 goat antirabbit, Invitrogen A11011) at 1:1000 dilution and 4′,6-diamidino-2-phenylindole counterstaining. Phospho-ATM foci were visualized with anti-phospho-ATM (serine1981) mouse monoclonal antibody (200-301-400; Rockland, Gilbertsville, PA). Costaining with PCNA was performed as described earlier. For costaining with 53BP1 foci, an anti-53BP1 rabbit polyclonal antibody (Abcam) was used. For all experiments, the number of foci per nucleus was determined by analyzing 100 to 300 nuclei per data point. For quantification of "induced foci," the number of

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foci per nucleus in untreated cells was subtracted from the foci number in treated cells and appropriate cutoffs between 10 and 20 foci per nucleus were chosen to determine the fraction of cells positive for foci formation depending on the particular endpoint. Observers were generally blinded as to whether cells had been treated.

Detection of RAD51 foci in tumor tissues has been described.[8] For visualization of γ-H2AX, cryosections were fixed with 2% paraformaldehyde for 15 minutes, permeabilized with 0.5% Triton X-100 for 5 minutes at room temperature, and stained with anti-γ-H2AX (phospho-S139) mouse monoclonal antibody (ab18311; Abcam) at 1:500 dilution at 4°C overnight, which was followed by incubation with secondary antimouse Alexa Fluor-488 antibody (Invitrogen, Carlsbad, CA) at 1:1000 dilution for 1 hour at room temperature. For PCNA staining, tissue sections were permeabilized first with 0.1% Tween-20 for 10 minutes and incubated with anti-PCNA rabbit polyclonal antibody (#ab2426; Abcam) at 1:200 dilution. For foci quantification, typically seven to 10 random images per data point were captured and 200 to 400 nuclei scored. Nuclei with at least two foci were considered positive. For three-dimensional rendering (Fig. 4*C*), a Z-series of images was taken for each treatment (SlideBook 5.0, IX81 Olympus confocal microscope; Olympus). The step size was 0.1 μm, and the number of planes was 40. The Z-series was deconvolved using the Constrained Iterative Method. The deconvolved Z-series was assembled into three-dimensional images using the three-dimensional Volume view option in SlideBook.

RESULTS

A NSCLC Cell Line Screen Reveals Putative HRR Defects

To identify NSCLC cell lines with impaired HRR, we sought to screen for the ability of cisplatin-treated cells to form subnuclear foci of RAD51, an established surrogate marker of HRR activity.[12,13] We first established that human cells with a known defect in the HRR pathway exhibit impaired RAD51 foci formation in the S-phase of the cell cycle, which is thought to reflect defective repair of replication forks that collide with drug-induced interstrand crosslinks.[2] Impaired RAD51 formation was not only seen in S-phase cells, but was also detectable in asynchronously growing cell populations at both 5 and 24 hours after start of drug treatment ($p < 0.05$) (Fig. 1*A*, Supplementary Fig. 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). Next, we screened a panel of 16 NSCLC cell lines for their proficiency to form RAD51 foci in response to cisplatin treatment. There was a range of foci-forming ability, with 4 of 16 cell lines (25%) demonstrating induced foci in 10% or less of cells suggesting a putative HRR defect (Fig. 1*B*). Cell cycle analysis indicated that the reduction in RAD51 foci formation was not due to a lower fraction of cells in S-phase (Supplementary Fig. 2, Supplemental Digital Content 1, http://links.lww.com/JTO/A384).

RAD51 Foci Defect Correlates with Crosslinker and PARP Inhibitor Sensitivity

HRR-defective cells are expected to be sensitive to crosslinking agents, such as cisplatin or mitomycin C (MMC), but also PARP inhibitors.[14] We first determined the cisplatin sensitivity of our cell line panel in a colony formation assay. Two RAD51 foci-impaired cell lines, H1915 and H2087, clearly demonstrated drug hypersensitivity (Fig. 2*A*), whereas the other two cell lines, H1563 and H2126, appeared to be relatively resistant to cisplatin. However, tumor cells could have acquired cisplatin resistance by other processes such as drug efflux mechanisms. We, therefore, also determined the MMC sensitivity of the four RAD51 foci-deficient cell lines

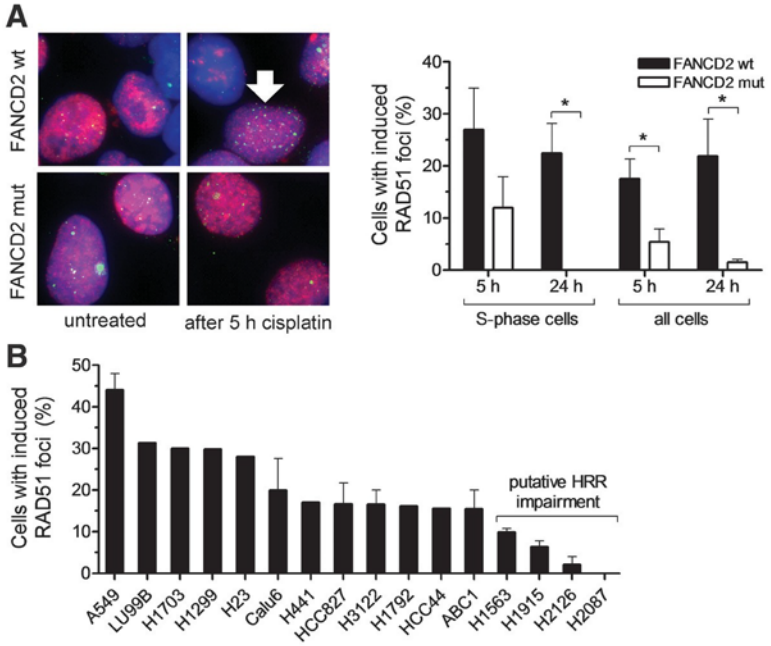

FIGURE 1. Monitoring the formation of subnuclear RAD51 foci in response to cisplatin treatment. (A) FANCD2-mutant (mut) PD20 fibroblasts with or without exogenous expression of wild-type (wt) FANCD2 were exposed to cisplatin (8 μM) for 1 hour and subjected to immunofluorescence stain- ing for the S-phase marker nuclear PCNA and RAD51 recombinase at 5 and 24 hours (h). Left panel shows representative images of costaining for PCNA (red) and RAD51 (green), in conjunction with 4′,6-diamidino-2-phenylindole (blue) counterstaining. Right panel shows fraction of cells with induced RAD51 foci in PCNA-positive S-phase cells or all cells. Bars represent mean with standard error based on three independent repeat experiments. Arrow indicates representative nucleus with intact RAD51 foci formation in an S-phase cell. *$p < 0.05$ (*t* test, two-tailed). (B) Analogous to panel A, 16 NSCLC cell lines were exposed to cisplatin and screened for induced RAD51 foci at 5 hours. Bars represent the fraction of cells with at least 15 induced RAD51 foci. NSCLC, non–small-cell lung cancer; PCNA, proliferating cell nuclear antigen.

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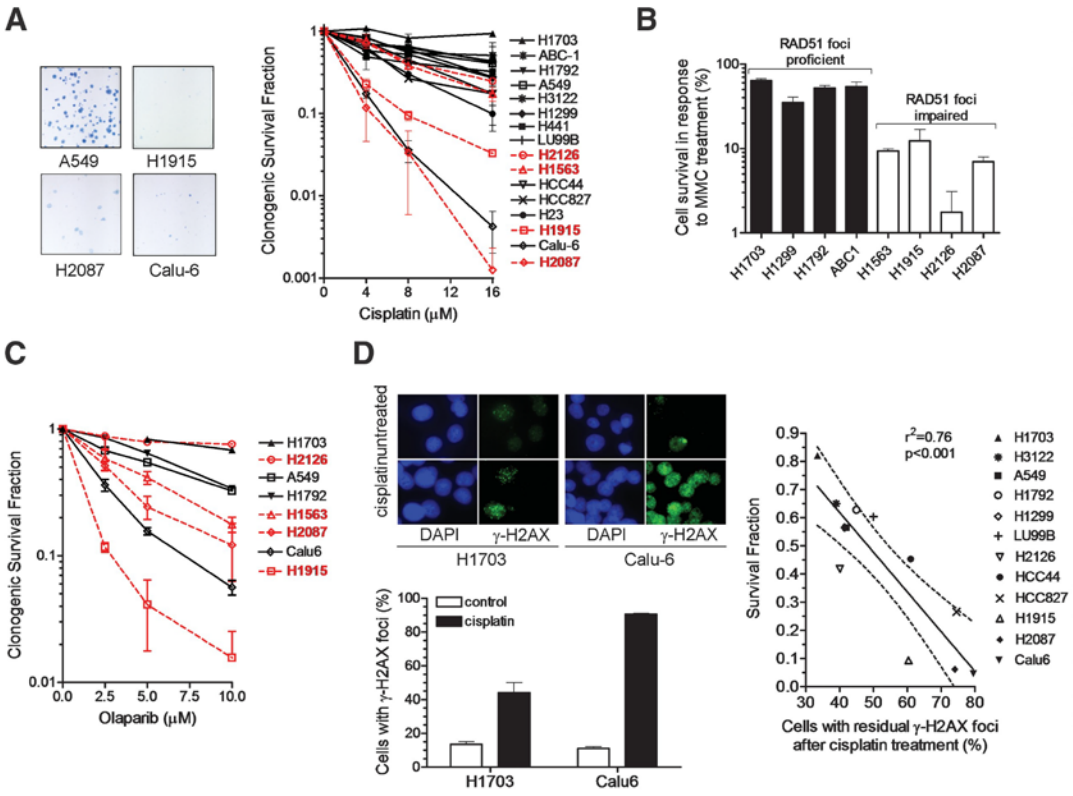

FIGURE 2. Sensitivity of NSCLC cell lines to DNA-damaging anticancer drugs. (A) Left panel shows representative examples of colony forming ability after treating A549 cells (200 cells seeded) or cisplatin-sensitive cell lines (2000–5000) with 16 μM cisplatin. Right panel, clonogenic survival fraction is plotted against cisplatin concentration. Data points represent mean with standard error based on at least two independent repeat experiments. Cell lines with putative RAD51 foci formation defects are highlighted in red. (B) Clonogenic survival fraction after treatment with 0.25 mg/ml mitomycin C for 1 hour is plotted for a subset of cell lines. (C) Clonogenic survival fraction after treatment with 10 μM of olaparib for 72 hours. Cell lines with putative RAD51 foci formation defects are highlighted in red. Bars represent mean with standard error based on at least two independent repeat experiments. (D) Left panels illustrate the difference in the formation of γ-H2AX foci at 24 hours after treatment of cisplatin-resistant H1703 and cisplatin-sensitive Calu-6 cells with 8 μM cisplatin. Right panel shows the correlation between clonogenic cell survival versus residual γ-H2AX foci present at 24 hours after cisplatin treatment with 8 μM. Dotted line reflects 95% confidence limits of the linear regression line. NSCLC, non–small-cell lung cancer.

and detected reduced clonogenic survival in all these cell lines compared with a set of RAD51 foci-proficient cell lines (Fig. 2*B*). Inhibition of PARP is known to cause synthetic lethality in HRR-deficient BRCA mutant cells.[2] Consistent with this, three of the RAD51 foci-deficient cell lines demonstrated increased sensitivity to olaparib (Fig. 2*C*). It remained unclear why H2126 cells are resistant to olaparib, though it is possible that the very slow growth of this cell line may have reduced the number of drug-induced breaks encountered during a cell cycle.

Interestingly, one seemingly RAD51-proficient cell line, Calu-6, was sensitive to cisplatin and olaparib (Fig. 2*A* and 2C). Because there could be a defect parallel to or downstream of RAD51 foci formation, we also assessed the levels of residual γ-H2AX foci as a marker of DSB.[15] After cisplatin treatment, we found high levels of DSB in Calu-6 cells, pointing toward an underlying recombinational repair defect (Fig. 2*D*). For all cell lines, there was an excellent correlation between the levels of residual DSB and cisplatin sensitivity (Fig. 2*D*). Thus, both RAD51 and γ-H2AX foci may be used to identify functionally relevant recombinational repair defects that are associated with cisplatin sensitivity.

ERCC1 is also involved in the repair and restart of replication forks, and is used as a biomarker of cisplatin resistance in the clinic.[1] We did not find a correlation between RAD51 foci induction and *ERCC1* gene expression, suggesting that ERCC1 is not a surrogate for RAD51 (Supplementary Fig. 3, Supplemental Digital Content 1, http://links.lww.com/JTO/A384).

Multiple Mechanisms Underlying Crosslinker Sensitivity in NSCLC Cells

Of the four cell lines with impaired RAD51 function, one cell line, H2126, has a genomic alteration in a pathway known to control HRR in response to replication stress,[16] i.e., a heterozygous mutation in the ATR kinase. H2087 cells harbor a mutation in the ATM kinase impairing phosphorylation at the S1981 site (Supplementary Fig. 4*A*, Supplemental

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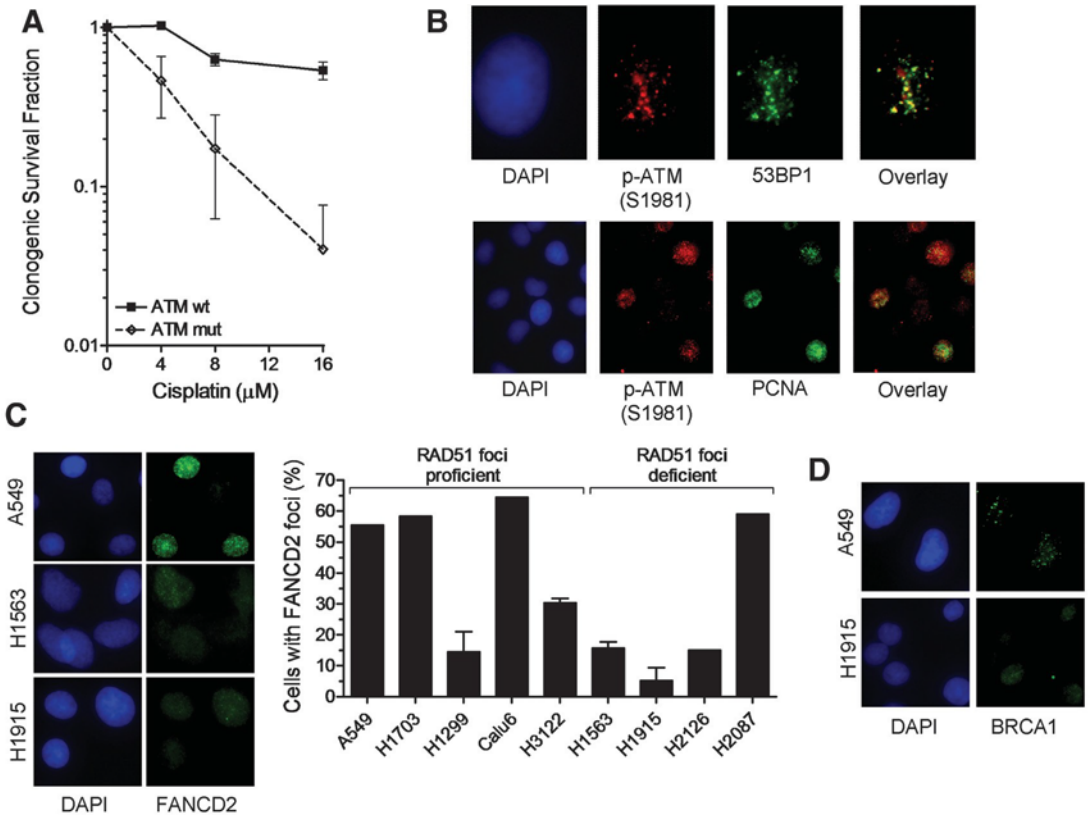

FIGURE 3. Pathway correlates of cisplatin sensitivity. (A) Clonogenic survival of human fibroblasts with wild-type (wt) or mutant (mut) ATM treated with varying doses of cisplatin for 1 hour. Data points represent mean with standard error based on three independent repeat experiments. (B) Representative images demonstrating colocalization of phospho-ATM foci with 53BP1 or PCNA at 24 hours after cisplatin treatment of A549 cells. (C) Analysis of FANCD2 foci formation at 5 hours in a subset of cell lines treated with 8 μM cisplatin. Bars represent the fraction of cells with 20 or more foci per nucleus. (D) Representative images illustrating the reduced ability of H1915 cells to form BRCA1 foci in response to cisplatin. PCNA, proliferating cell nuclear antigen.

Digital Content 1, http://links.lww.com/JTO/A384). ATM is known to be involved in nonhomologous end-joining and HRR after the induction of DSB by ionizing radiation but has not been previously implicated in cisplatin resistance.[17] Follow-up analysis in several cell systems established that ATM is required for cellular cisplatin resistance (Fig. 3*A*, Supplementary Fig. 4*B*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). The kinetics of ATM activation followed the time course of DSB induction (Supplementary Fig. 4*C*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). Phosphorylated ATM co-localized with DSB (Fig. 3*B*), was detected during DNA replication within 6 hours after cisplatin treatment (Fig. 3*B*, Supplementary Fig. 4*D*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384), and formed in a manner that appeared at least partially dependent on ATR (data not shown), consistent with a role of ATM at stalled replication forks.[18]

For RAD51 foci-impaired cell lines H1563 and H1915, no preexisting genomic alteration in a putative HRR pathway was known. We thus screened for reduced expression of components of the FA/BRCA pathway and detected decreased FANCD2 and BRCA1 expression, respectively (data not shown), consistent with previous reports showing altered expression of these genes in lung cancer.[3,4] Significantly, we found a reduced ability to form FANCD2 foci in both cell lines (Fig. 3*C*). In addition, in H1915 cells, there was reduced foci formation of BRCA1 (Fig. 3*D*), which is upstream of FANCD2 foci formation.[19]

Unexpectedly, our analysis also revealed that H1299 cells exhibit reduced expression of FANCF (Supplementary Fig. 5*A*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384), which was associated with impaired FANCD2 foci formation (Fig. 3*C*) and mono-ubiquitination of FANCD2 (Supplementary Fig. 5*B*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). However, these cells were not crosslinker sensitive (Fig. 2*A* and 2*B*), likely due to unusually high levels of RAD51 protein (Supplementary Fig. 5*C*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384) that may act in a compensatory manner.[20,21]

An Ex Vivo Biomarker Assay to Monitor the Effects of Anticancer Agents in Live Tumor Explants

Our cell line work indicated that chemosensitivity is correlated with an impaired ability to induce RAD51

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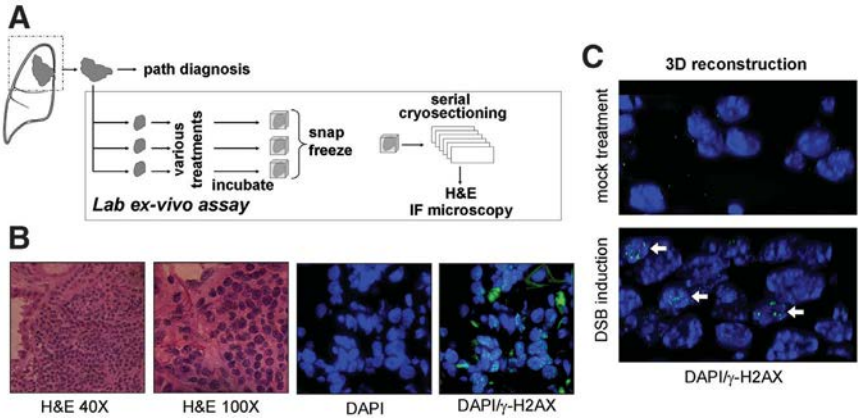

FIGURE 4. Ex vivo foci assay in NSCLC explants. (A) Surgical specimens from untreated patients were incubated under standard cell culture conditions and exposed to 8 μM cisplatin, 10 Gy radiation, or mock treatment ex vivo. Specimens were snap frozen after 5 or 24 hours and subjected to cryosectioning at a later time. (B) Serial cryoslides were analyzed by H&E staining to identify viable tumor and immunofluorescence (IF) microscopy to visualize repair protein foci. (C) Three-dimensional rendering of tissue nuclei counterstained with γ-H2AX 5 hours after mock treatment or irradiation ex vivo. NSCLC, non–small-cell lung cancer.

foci after drug treatment (Fig. 1, Supplementary Fig. 6*A*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384), but not with the baseline number of foci in untreated cells (Supplementary Fig. 6*A*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). Thus, assaying RAD51 foci on archived tumor samples (from pretreatment biopsies) was not expected to be informative.

We, therefore, adapted an ex vivo biomarker assay previously tested in breast cancer to determine if impaired HRR activity can be detected in NSCLC.[8] Fresh tumor tissues from patients with untreated NSCLC were collected and within 90 minutes of removal from the patient exposed to cisplatin or radiation in the laboratory (Fig. 4*A*). Tissues were incubated in parallel for 5 hours before snap freezing. Serial cryosections were analyzed for viable tumor by H&E staining and nuclear protein foci using immunofluorescence microscopy (Fig. 4*B*). Damage-induced γ-H2AX foci were readily visualized after irradiation or cisplatin treatment (Fig. 4*B* and 4*C*, Supplementary Fig. 6*B*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384).

We assessed a total of 13 NSCLC explants for cellular proficiency to form RAD51 foci in response to cisplatin treatment (Fig. 5*A*). Similar to the cell line data, there was a range of foci induction with levels overall lower than in cell lines, i.e., on average, only 5.8% of tumor cells (95% CL, 3.0–8.7%) exhibited RAD51 foci. In 2 of 13 tumors (15%), i.e., explants L006 and L007, very few RAD51 foci (in <1% of cells) were seen in the cisplatin-treated specimens, which is indicative of impaired HRR activity. The overall lower fraction of cells with RAD51 foci in explants compared with the cell lines was explained by a several fold fraction of cells in S-phase (Fig. 5*B*, Supplementary Fig. 6*C*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). Importantly, tumors with putative HRR defects displayed an S-phase fraction comparable with RAD51 foci-proficient tumors, as illustrated in Figure 5*C*. In addition, low RAD51 foci levels did not result from a lack of DNA damage induction, as shown by the ability of cisplatin to generate γ-H2AX foci in these explants (Fig. 5*D*, Supplementary Fig. 6*B*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). Taken together, these data demonstrate the feasibility of monitoring a drug-induced cellular DNA damage response in live tumor explants ex vivo.

DISCUSSION

The identification of HRR-deficient tumors is a major challenge in cancer research, in particular when taking into account the complexity of the DNA damage response network.[2] In addition, assessing the expression of individual network components is unlikely to reveal the overall incidence of defects that can occur anywhere in the network. Finally, it is not established whether reduced expression of genes involved in HRR translates into functional repair impairment.

There has been increasing interest in determining HRR activities in cancers by assaying for the subnuclear location of central pathway components, such as RAD51, BRCA1, and FANCD2, and unrepaired DSB marked by γ-H2AX.[8,22,23] The advantage of using foci as biomarkers is that they can capture repair defects due to various mechanisms such as gene mutations, epigenetic changes, or alterations in signal transduction pathways.[2] Moreover, they provide a global measurement of network function without needing to know the identities of all the components, many of which remain unknown.

Here, we report for the first time the presence of impaired RAD51 foci formation in 4 of 16 NSCLC cell lines (25%). All four cell lines were MMC hypersensitive (Fig. 2*B*), whereas the sensitivity profile for cisplatin and olaparib was more heterogeneous (Fig. 2*A* and 2*C*). Although the exact reason for this differential effect remains to be elucidated, it is conceivable that residual HRR function in some cell lines is sufficient to cope with the consequences of intrastrand DNA damage associated with olaparib and/or cisplatin treatment. In contrast, a higher percentage of the DNA damage generated by MMC consists of damage affecting both DNA strands, i.e.,

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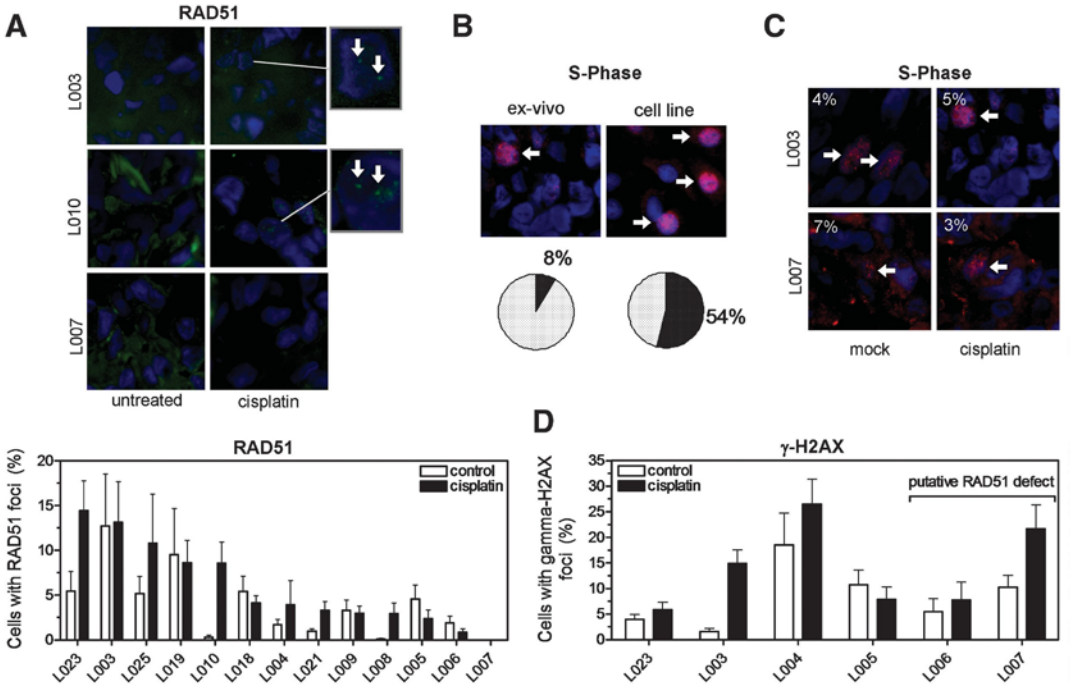

FIGURE 5. Detection of cisplatin-induced RAD51 foci in live NSCLC explants. (A) Upper panel shows representative images demonstrating subnuclear RAD51 foci after 5 hour incubation after cisplatin. Lower panel displays the fraction of nuclei with RAD51 foci in a panel of 13 tumor explants. Bars represent mean with standard error. (B) Illustration of S-phase fraction in representative tumor tissue versus A549 cells using PCNA/DAPI counterstaining (see also Supplementary Fig. 6*C*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). (C) Illustration of PCNA-positive S-phase fraction in RAD51 foci-proficient versus -deficient tumors. (D) Quantification of fraction of cells with cisplatin-induced γ-H2AX foci at 5 hours. Bars represent average fraction of nuclei with at least two foci ± standard error based on 8 to 10 random images and 200 to 400 nuclei per data point. DAPI, 4′,6-diamidino-2-phenylindole; NSCLC, non–small-cell lung cancer; PCNA, proliferating cell nuclear antigen.

interstrand crosslinks, which places a much greater burden on the cellular DNA repair response than the damage resulting from the other two compounds.[2] Thus, assaying for tumors with defective RAD51 formation should at least enrich for those that may respond to cisplatin or PARP inhibitors.

In addition, we detected a strong correlation between persisting γ-H2AX foci and cisplatin sensitivity. These data and the identification of one RAD51-proficient cell line, Calu-6, which demonstrates high γ-H2AX levels and cisplatin sensitivity, support the notion that joint assessment of RAD51 and γ-H2AX foci may be sufficient to reliably identify platinum- and perhaps also olaparib-sensitive tumors. Importantly, RAD51 foci form independently of ERCC1,[24] and we do not find a correlation between foci induction and ERCC1 expression (Supplementary Fig. 3, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). Whether ERCC1 in conjunction with RAD51 and γ-H2AX foci constitutes a useful biomarker set requires further study.

Our data indicate that foci responses yield information that can complement or supersede genomic information. For example, ATM-mutant H2087 cells were sensitive to cisplatin and displayed impaired RAD51 foci (Fig. 3), even though ATM has not been previously implicated in cellular cisplatin resistance. As a fraction of lung adenocarcinoma (7%) harbor nonrecurrent mutations throughout the *ATM* gene,[6] the assessment of foci responses in these tumors may provide information on the functional significance of these alterations. As another example, FANCF hypermethylation was reported in 14% of NSCLC, but the functional impact of these epigenetic events has remained unknown.[4] Here, we found that the often studied H1299 cell line displays suppressed FANCF expression and impaired downstream FANCD2 function (Fig. 3, Supplementary Fig. 5, Supplemental Digital Content 1, http://links.lww.com/JTO/A384), yet retains RAD51 function and drug resistance (Fig. 2), which highlights the usefulness of RAD51 and γ-H2AX foci as biomarkers of functionally relevant repair defects and drug sensitivity.

How can foci assays in cell lines be translated into patients? It is important to appreciate that the functional status of HRR is typically revealed only when cells are exposed to DNA damage.[8] Assessing foci responses in live tumors would require a repeat biopsy after initial administration of treatment, which is a challenging undertaking. Alternatively, pretreatment biopsies can be interrogated for their functional HRR status to select the appropriate treatment regimen for a given patient (Fig. 4*A*). We report here for the first time that cisplatin induces a damage response in NSCLC explants from untreated patients (Fig. 5*D*, Supplementary Fig. 6*B*, Supplemental Digital Content 1, http://links.lww.com/JTO/A384). At least two of 13 tumors analyzed in this fashion demonstrated low RAD51 foci levels consistent with a functional HRR defect (Fig. 5*A*). Whether patients with these

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tumors indeed derive benefit from treatment with cisplatin or a PARP inhibitor remains to be determined.

Although this type of functional ex vivo foci assay represents a potentially powerful tool for the detection of preexisting and clinically relevant defects within the complex HRR pathway, several technical challenges remain, including (1) potential intratumoral heterogeneity in foci responses, (2) low fraction of cells in S-phase, compared with the cell lines, necessitating costaining techniques to detect replication-associated RAD51 foci, (3) need for quantification and automation of foci scoring, and (4) potential changes in hypoxia/reoxygenation upon removal of the tumor tissue from the patient and incubation in the laboratory at 20% oxygen. Data from our laboratory and by Vaira et al.[25] indicate that adequately cultured tumor explants are viable for at least 24 hours and up to 5 days, offering a promising avenue for assessing not only foci responses but also surrogate endpoints of cell fate such as apoptosis or senescence, thereby allowing pharmacodynamic profiling of human tumors.

In conclusion, it should be possible to use the ability of cells to form repair foci as a functional biomarker of the integrity of the HRR pathway. The absence of repair foci induction, coupled with a persistence of DSB markers such as γ-H2AX, would be indicative of chemosensitivity or PARP inhibitor sensitivity. One can envision developing mechanism-based "HRR foci signatures" that reflect nodal points in the HRR pathway or network of associated DDR proteins. Such a foci signature likely will include at a minimum BRCA1, FANCD2, RAD51, and γ-H2AX to capture the multiple deficiencies that seem to underlie crosslinker and PARP inhibitor sensitivity in NSCLC.

Alterations in HRR in tumors can be therapeutically targeted by novel approaches such as PARP inhibitors and traditional DNA-damaging chemotherapeutics or radiation. The efforts to exploit preexisting HRR defects or disrupt proficient or hyperactive HRR in malignancies are in their infancy, but hold great promise to broadly impact cancer therapies in the very near future.[2] As our understanding of the regulation of HRR pathways in normal and malignant cells deepens, various rational treatment strategies are likely to materialize.[26]

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Supplementary Figures

FIGURE S1. Representative images to illustrate RAD51 foci induction in FANCD2 wild-type (wt) and mutant (mut) cells. Higher magnification is shown in Figure 1A. Arrows indicate cisplatin-induced foci. Blue, DAPI; red, PCNA; green, RAD51.

FIGURE S2. Cell-cycle distributions were determined using standard ethanol fixation and propidiumiodide (Sigma-Aldrich) staining followed by flow cytometry at a core facility at the Ragon Institute at MGH.

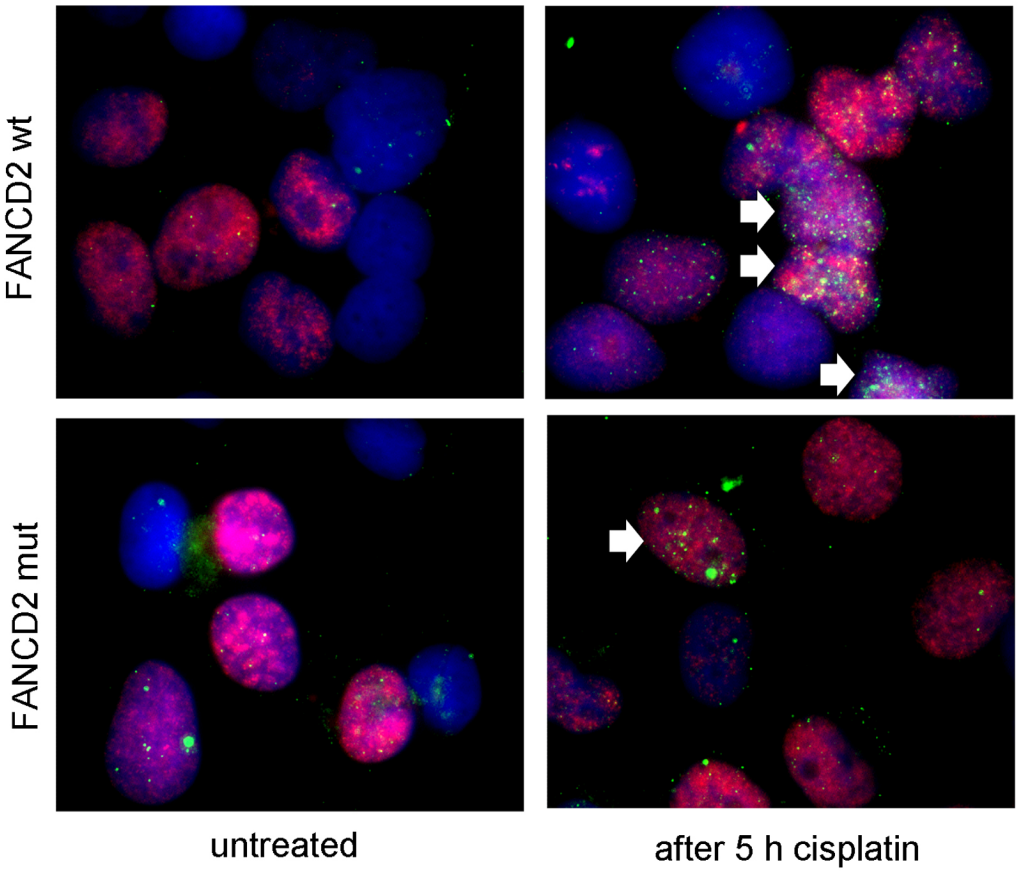
FIGURE S3. Correlation of ERCC1 gene expression with cisplatin-induced RAD51 foci.
Linear regression line is shown. Gene expression data were obtained from the publicly available Cancer Cell Line Encyclopedia (CCLE).
http://www.broadinstitute.org/ccle/home

FIGURE S4. Role of ATM in cisplatin resistance.
A, Representative images illustrating reduced ATM phosphorylation in H2087 cells 24 hours after treatment with 8 µM cisplatin. Phospho-ATM was visualized with mouse anti-ATM pS1981 monoclonal antibody (Rockland #200-301-400) at a 1:100 dilution. B. Clonogenic cell survival following cisplatin treatment. Left panel, A549 cells were mock treated or treated with the ATM kinase inhibitor KU55933 at 20 µM (Chemdea). Right panel, mouse embryonic fibroblasts (MEFs) with the indicated genotypes. Data represent means +/- standard error based on 2-3 biological repeats. C, Time course of γ-H2AX and p-ATM (S1981) foci in A549 and Calu-6 cells following treatment with 8 µM cisplatin for 1 hour. D, Co-staining of PCNA and p-ATM foci in A549 cells treated with 8 µM cisplatin. To eliminate non-chromatin bound PCNA, cells were permeabilized first with 0.5% Triton-X, 20mM HEPES, 50mM NaCl, 3mM KCl, 300mM Sucrose on ice for 5 minutes, followed by fixation with 2% paraformaldehyde at room temperature for 20 minutes and 100% methanol at –20°C for 10 minutes. Cells were exposed to primary anti-pATM pS1981 antibody (see above) and anti-PCNA (Rabbit polyclonal antibody, Abcam ab2426) at 1:200 dilution in 2% BSA, 0.1% Triton X-100 in PBS, for 2 hours at room temperature. The percentage of nuclei with either PCNA or p-ATM or the combination of both was scored at the time points indicated. At least 100 nuclei were counted per data point. Bars represent the mean of 2 biological replicates.

FIGURE S5. Analysis of Fanconi Anemia (FA) pathway function in H1299 cells.
A, Whole cell lysates from exponentially growing NSCLC cells were subjected to Western blotting with anti-FANCF rabbit polyclonal antibody (Sigma-Aldrich, SAB1101098) at a 1:500 dilution. Standard beta-actin (Sigma-Aldrich) was used as a loading control. B, Exponentially growing cell lines were exposed to 40 ng/mL MMC for 24 hours. Whole cell lysates were subjected to Western blotting with anti-FANCD2 (FI17) mouse monoclonal antibody (Santa Cruz, sc 20022) at 1:100 dilution. In order to optimize separation of the damage-induced mono-ubiquitinated form of FANCD2 (L) from the unmodified form (S), a 3-8% TrisAcetate gradient gel (BioRad) was used. C, Western blot for RAD51 using anti-Rad51 mouse monoclonal antibody (GeneTex, GTX70230) at 1:1,000 dilution.

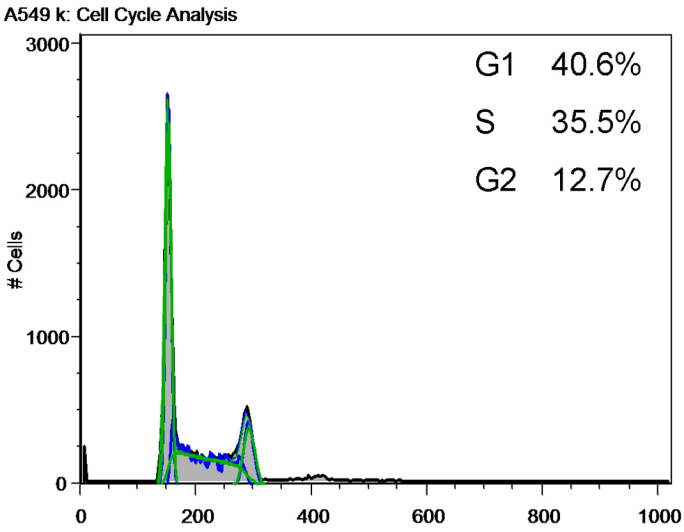
FIGURE S6. Translation of cell line findings to tumor tissues.
A, Correlation of Rad51 foci formation with clonogenic cell survival after treatment with 8 mM cisplatin. Upper panel correlates survival with “induced” RAD51 foci, i.e., fraction of nuclei with at least 15 RAD51 foci following cisplatin treatment minus fraction of nuclei with at least 15 RAD51 foci in untreated cells. Lower panel shows lack of correlation of survival with “baseline” fraction of nuclei with at least 15 RAD51 foci without cisplatin treatment. B, Representative images demonstrating induction of γ-H2AX foci in NSCLC explants following 5 hours after irradiation (IR) with 10 Gy or treatment with 8 μM cisplatin. Arrows indicate foci, which are less frequent and more difficult to discern after cisplatin than after radiation treatments. C, Estimation of the fraction of cells in S-phase. Upper panel, the fraction of nuclei with FANCD2 foci (which almost exclusively form in S-phase) and PCNA is shown for A549 cells. Approximately 50% of cells are in S-phase. Lower panel, semi-quantification of nuclei with at least 2 FANCD2 foci in untreated NSCLC explants. Percentages represent averages based on 7-10 random images and 200-400 nuclei.

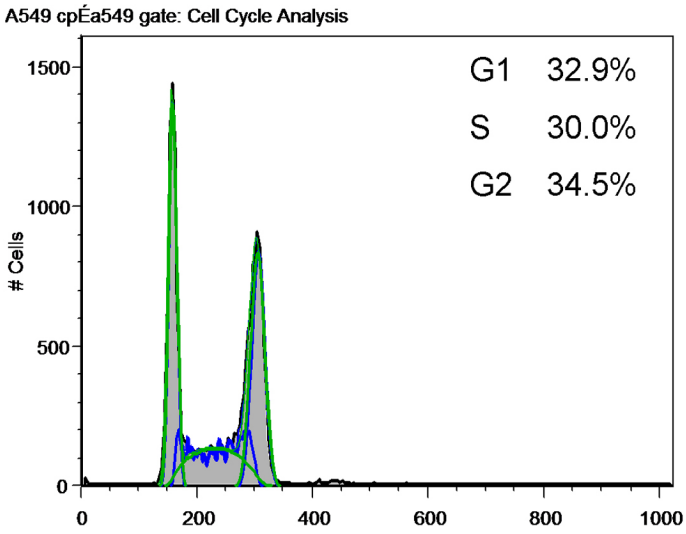
Suppl. Fig. S1



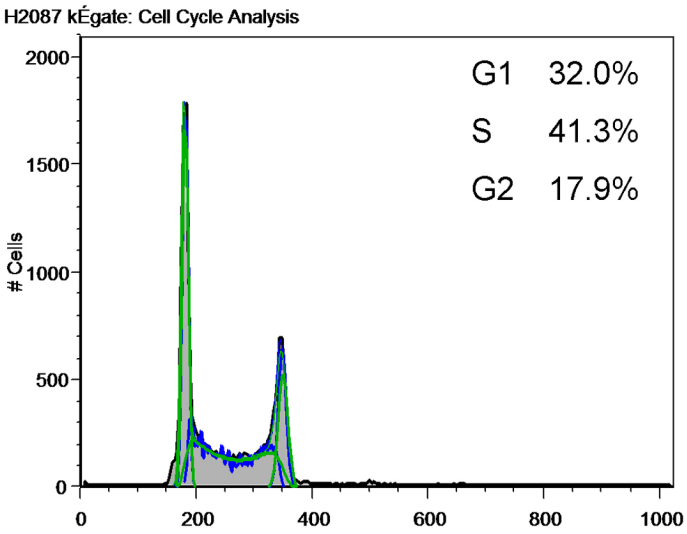
Suppl. Fig. S2

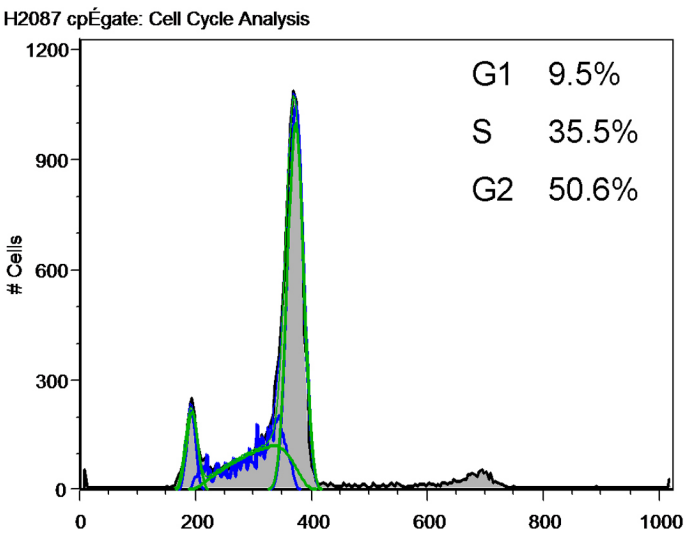
A549



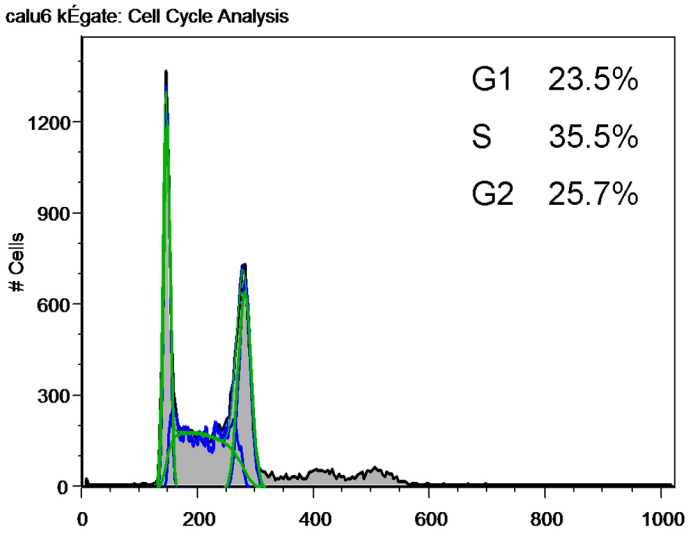


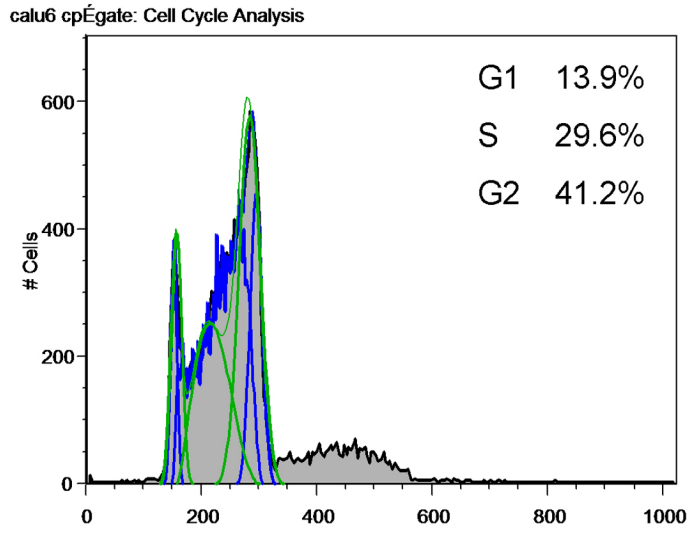
H2087





Calu-6

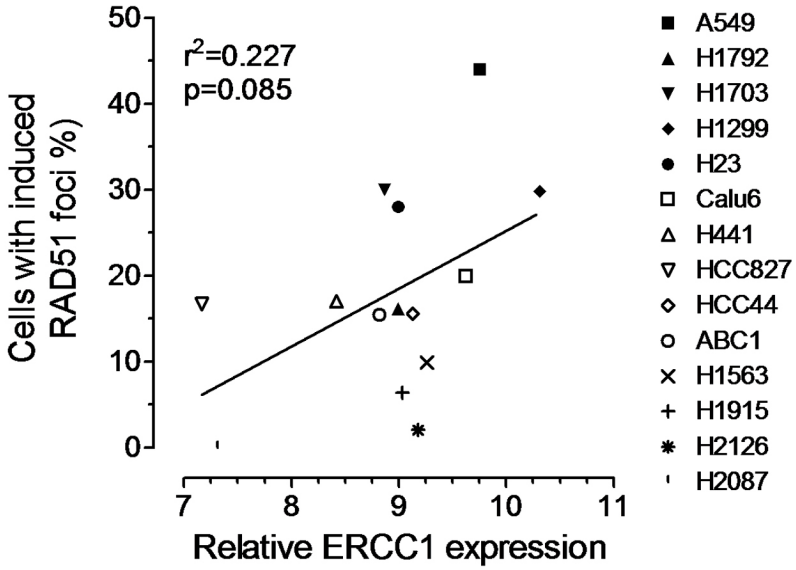




untreated

cisplatin

Suppl. Fig. S3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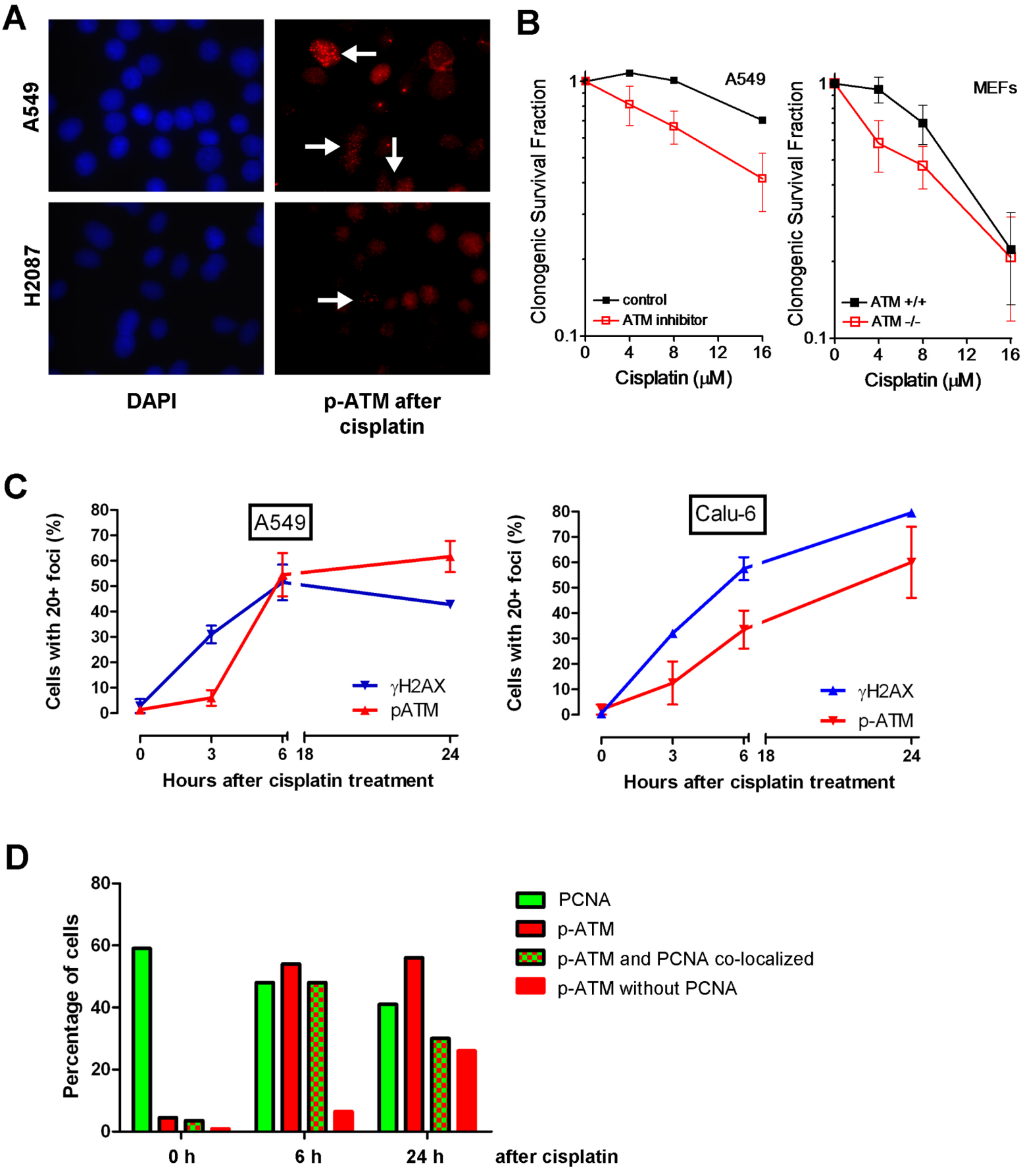
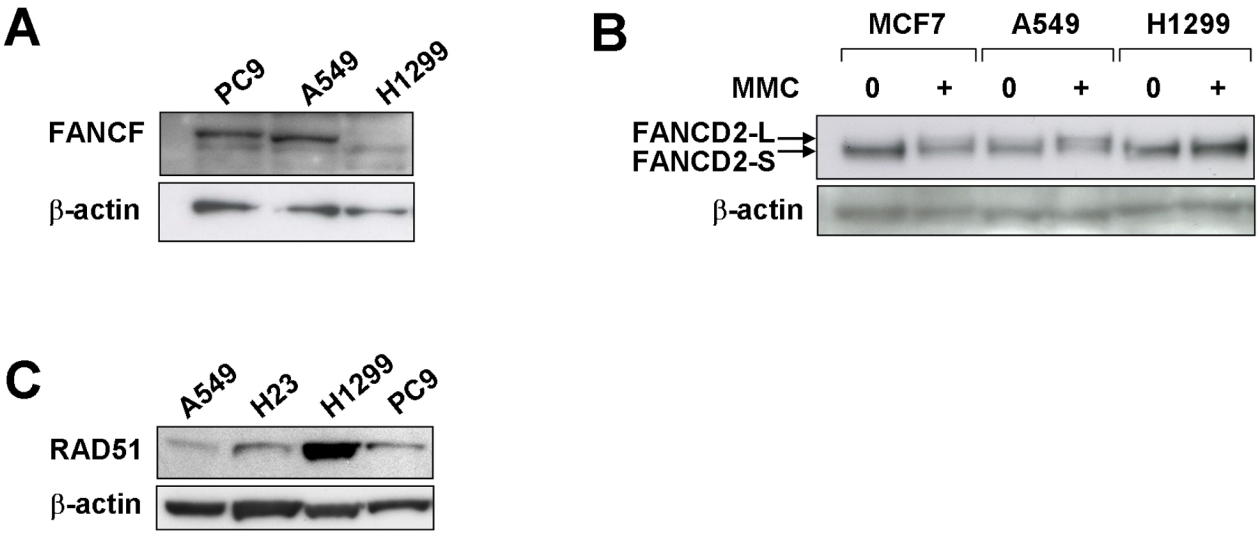
p=0.085
Cells with induced RAD51 foci %)
Relative ERCC1 expression
0
10
20
30
40
50
7
8
9
10
11
A549
H1792
H1703
H1299
H23
Calu6
H441
HCC827
HCC44
ABC1
H1563
H1915
H2126
H2087

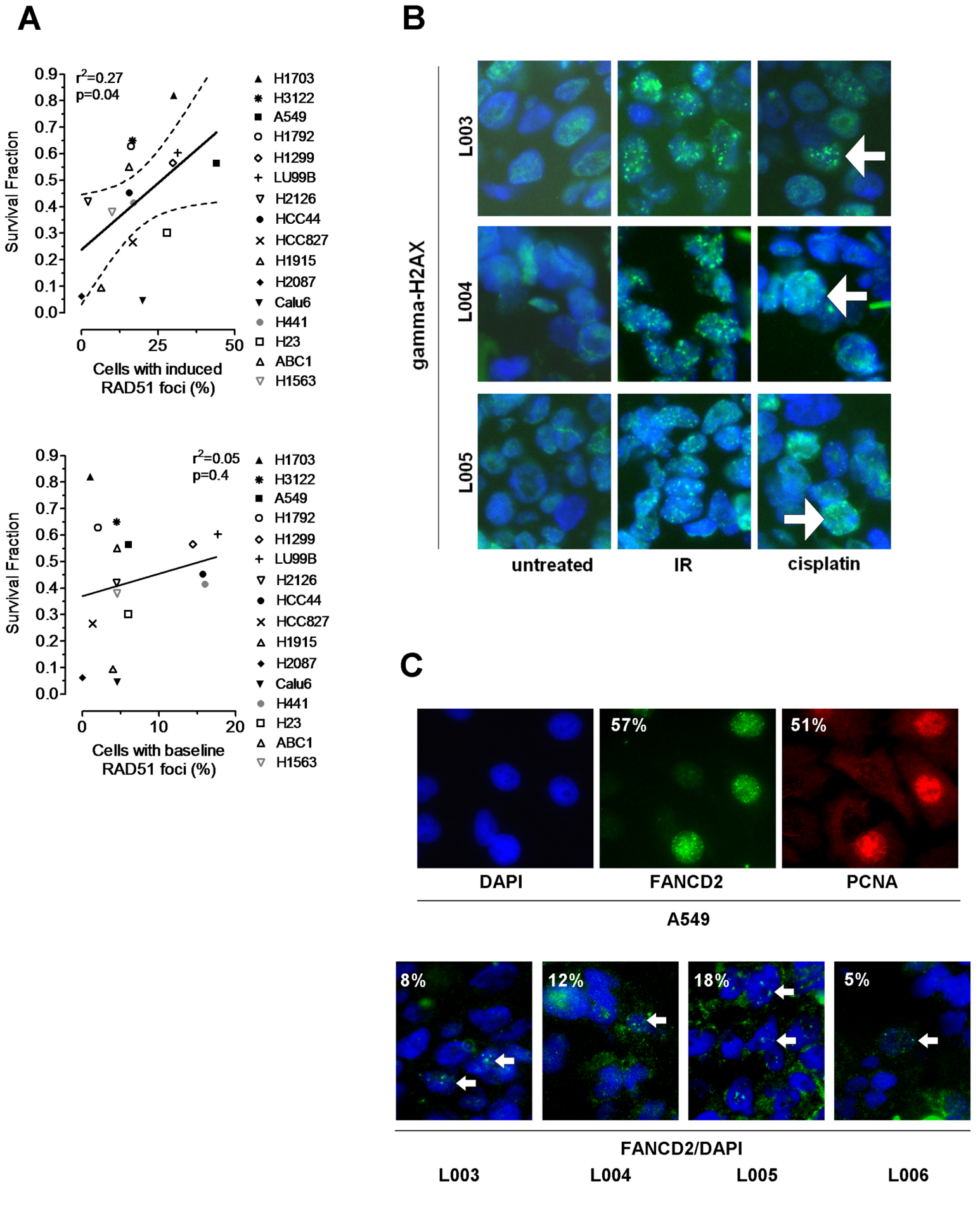
Suppl. Fig. S4


A
A549
H2087
DAPI
p-ATM after cisplatin
B
Clonogenic Survival Fraction
A549
control
ATM inhibitor
Cisplatin (μM)
MEFs
ATM +/+
ATM -/-
C
A549
Calu-6
Cells with 20+ foci (%)
γH2AX
pATM
p-ATM
Hours after cisplatin treatment
D
Percentage of cells
PCNA
p-ATM
p-ATM and PCNA co-localized
p-ATM without PCNA
0 h
6 h
24 h
after cisplatin

Suppl. Fig. S5


A
PC9
A549
H1299
FANCF
β-actin
B
MCF7
A549
H1299
MMC
0
+
0
+
0
+
FANCD2-L
FANCD2-S
β-actin
C
A549
H23
H1299
PC9
RAD51
β-actin

Suppl. Fig. S6


A
r²=0.27
p=0.04
Survival Fraction
Cells with induced RAD51 foci (%)
H1703
H3122
A549
H1792
H1299
LU99B
H2126
HCC44
HCC827
H1915
H2087
Calu6
H441
H23
ABC1
H1563
r²=0.05
p=0.4
Cells with baseline RAD51 foci (%)
B
gamma-H2AX
L003
L004
L005
untreated
IR
cisplatin
C
57%
51%
DAPI
FANCD2
PCNA
A549
8%
12%
18%
5%
FANCD2/DAPI
L003
L004
L005
L006

View Letter

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Date: Nov 19, 2012
To: "Henning Willers" hwillers@partners.org
cc: jettj@njhealth.org, gabriella.sozzi@istitutotumori.mi.it
From: "Journal of Thoracic Oncology" thoraciconcology@earthlink.net
Subject: JTO Decision

Nov 19, 2012

RE: JTO-D-12-00496R1, entitled "Detection of Impaired Homologous Recombination Repair in NSCLC Cells and Tissues"

Dear Dr. Willers,

I am pleased to inform you that your work has now been accepted for publication in Journal of Thoracic Oncology. All manuscript materials will be forwarded immediately to the production staff for placement in an upcoming issue.

Thank you for submitting your interesting and important work to the journal.

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With Kind Regards,

Gabriella Sozzi
Associate Editor
Journal of Thoracic Oncology

Close

Zusammenfassende Darstellung der Publikation

Einführung und Hintergrund

Das Bronchialkarzinom ist eine der häufigsten Todesursachen in Deutschland und die führende Ursache Krebs-assoziierter Sterbefälle. Mehr als eine Millionen Fälle des Bronchialkarzinoms werden weltweit jedes Jahr diagnostiziert (1,2). Mit einem Anteil von 85% an allen Lungenkrebsfällen ist das nicht-kleinzellige Bronchialkarzinom (non small cell lung cancer, NSCLC) die häufigste Form dieser Erkrankung (3). Eine Vielzahl dieser Tumoren wird erst im Stadium III und höher diagnostiziert und ist dann chirurgisch nicht mehr zu kurieren. Die wichtigste Säule der Therapie von diesen Patienten mit lokal fortgeschrittenem oder metastasiertem Lungenkrebs stellt heutzutage die Platin-basierte Chemotherapie dar. Diese hat jedoch nur eine Ansprechrate von 15-35% und die 5-Jahres Überlebensrate beträgt lediglich 14%, woraus sich ein dringender Bedarf bezüglich der Erforschung von neuartigen Wegen der Therapieoptionen ergibt (4-6).
Neueste Erkenntnisse weisen darauf hin, dass zelluläre Defekte in DNA-Reparaturwegen, welche zu genomischer Instabilität und somit zur malignen Transformation einer Zelle führen können, jedoch in gleicher Weise auch die betroffenen Zellen für exogene DNA-Schädigung durch Chemotherapeutika oder Bestrahlung sensibilisieren (7-9). Dies konnte für den Signalweg der Homologen Rekombination (HR) gezeigt werden, einem wichtigen zellulären Mechanismus zur fehlerfreien Reparatur von DNA Doppelstrangbrüchen (DSB) und Blockaden der Replikation wie sie z. B. durch platinhaltige Substanzen ausgelöst werden (10,11).
Ein wichtiges Merkmal der Homologen Rekombination und anderer DNA-Reparaturmechanismen ist die räumliche und zeitliche Organisation der in dem jeweiligen Signalweg involvierten Proteinfaktoren am Ort von beschädigter DNA (12). Somit kommt es als Antwort auf eine DNA-Schädigung zu einer Akkumulation von Proteinen, die sich im Zellkern zu mikroskopisch sichtbaren „Foci“ formieren. In nicht geschädigten Zellen ist deren Anzahl gering. Die Fähigkeit einer Zelle zur Formation dieser „Reparaturfoci“ kann somit dazu dienen, Auskunft über Funktionalität oder Defekt des Signalweges von DNA-Reparaturmechanismen zu erhalten und könnte sich daher als prädiktiver Biomarker zur Einschätzung von Sensibilität oder Resistenz von Tumorzellen gegenüber Cisplatin und anderen Chemotherapeutika eignen.

In dieser Arbeit werden funktionell relevante Defekte im Signalweg der Homologen Rekombination in verschiedenen nicht-kleinzelligen Lungenkrebszellen- und Gewebe untersucht. Dazu wird, als Antwort auf DNA-Schäden, die Fähigkeit von Tumorzellen zur Formation vom Reparaturfoci (v.a. Rad51und γ-H2AX) untersucht, welche eine zentrale Rolle im Signalweg der Homologen Rekombination bzw. der Reparatur von DNA-Doppelstrangbrüchen spielen (12,13).
Dies dient dem Ziel zelluläre Tumormarker zu identifizieren, welche vorhersagen können, ob ein Tumor mit hoher Wahrscheinlichkeit auf eine platinbasierte Chemotherapie ansprechen wird. Klinisch könnten dadurch in Zukunft Patienten ausgeschlossen werden, die voraussichtlich nicht von einer solchen Therapie profitieren, sodass ihnen die schweren Nebenwirkungen einer platinbasierten Chemotherapie wie Nephro- und Neurotoxizität erspart blieben.

Methoden:

In dieser Arbeit wurde das klonogene Überlebensverhalten von 16 nicht-kleinzelligen Bonchialkarzinomzelllinien in Abhängigkeit von einer Behandlung mit Cisplatin, Mitomycin C (MMC) und dem PARP-Inhibitor Olaparib anhand von Koloniebildungs-Tests (Colony Survival Assays) beurteilt. Durch immunzytochemische Fluoreszenzmikroskopie wurden die Zelllinien auf ihre Fähigkeit zur Bildung von Reparatur-assoziierten Foci wie BRCA1, FANCD2, RAD51 und γ-H2AX hin untersucht. Zellzyklusanalysen wurden mittels fluoreszenz-aktivierter Zellsortierung (FACS) durchgeführt. Die Funktionalität des Fanconi-Anämie Signalwegs (FA) in den Tumorzellen wurde anhand von Western Blots untersucht. Dreizehn Tumorbiopsien von Patienten mit nicht-kleinzelligem Bronchialkarzinom wurden ex vivo mit Cisplatin behandelt und darauffolgend analysiert.

Ergebnisse:

Um NSCLC-Zellinien mit Defekten im Signalweg der Homologen Rekombination zu identifizieren, wurden diese mit Cisplatin behandelt und anschließend die Fähigkeit der Zellen zur Bildung von RAD51-Foci untersucht, einem Marker zur Detektion der Aktivität der Homologen Rekombination während DNA-Reparatur in Tumorzellen (14,15).
Es konnte gezeigt werden, dass humane Tumorzellen, welche einen bekannten Defekt im Signalweg der Homologen Rekombination aufweisen, nämlich ein mutiertes FANCD2-Gen, in der S-Phase des Zellzyklus eine herabgesetzte Fähigkeit zur Bildung von RAD51 Foci besitzen. Dies wird als Unvermögen der Zelle gedeutet, eine adäquate DNA-Reparatur durchzuführen, nachdem während der DNA Replikation Replikationsgabeln mit Zytostatika-induzierten DNA-Querverknüpfungen (sog. interstrand crosslinks, ICL) kollidiert sind (12). Eine herabgesetzte Induktion von RAD51-Foci konnte nicht nur in Tumorzellen gezeigt werden, welche sich in der S-Phase des Zellzyklus befanden, sondern auch in asynchron wachsenden Zellpopulation sowohl 5 als auch 24 Std. nach einer Behandlung mit Cisplatin. (**Abb.1**).

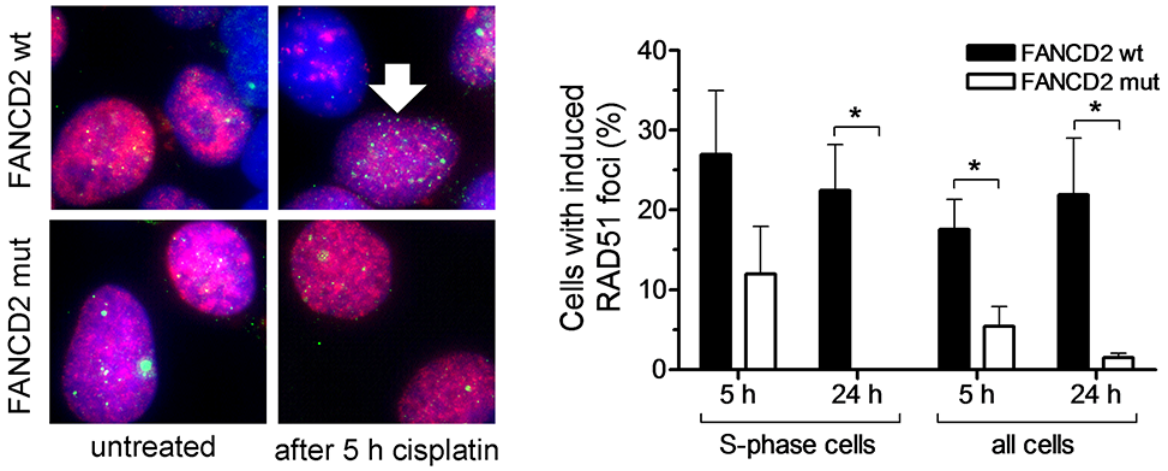

Abb. 1: Links: Fibroblasten mit mutiertem FANCD2-Gen (mut) und solche mit Wildtyp FANCD2 (wt) wurden für 1 Std. mit Cisplatin (8µM) behandelt und per Immunfluoreszenzmikroskopie auf den S-Phase-Marker PCNA (grün) und RAD51 (rot) angefärbt. Gegenfärbung des Zellkerns mit DAPI. Rechts: Prozentualer Anteil der wt und mut FANCD2 Zellen mit induzierten RAD51-Foci in PCNA-positiven S-Phase Zellen und allen Zellen. Die Balken stellen Mittelwerte mit Standardabweichung dar, berechnet aus mind. drei voneinander unabhängigen Versuchen.

Wir untersuchten 16 Zelllinien von nicht-kleinzelligen Bronchialkarzinomen auf deren Fähigkeit hin zur Bildung von RAD51-Reparaturfoci nach Behandlung mit Cisplatin. Bei vier der 16 untersuchten Zelllinien (25%) konnte eine herabgesetzte Formierung von RAD51-Foci beobachtet werden, gemessen daran, dass Reparaturfoci in lediglich 10% oder weniger der Zellen gebildet wurden, sodass ein Defekt im Signalweg der Homologen Rekombination in diesen Zelllinien vermutet werden kann (**Abb.2**).

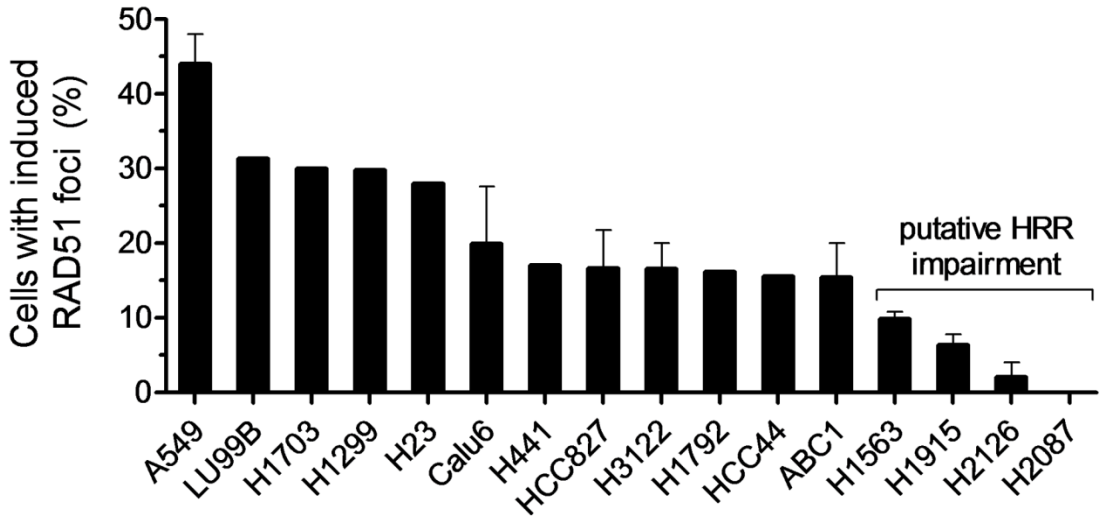

Abb.2: Analog zu Abb.1 wurden 16 NSCLC Zelllinien mit Cisplatin behandelt und auf deren Fähigkeit zur Bildung von RAD51-Reparaturfoci nach 5Std. untersucht. Die Balken repräsentieren den prozentualen Anteil von Zellen mit mindestens 15 induzierten RAD51-Foci.

Anhand von Zellzyklusanalysen wurde gezeigt, dass ein Unvermögen zur Bildung von Reparaturfoci der Zelllinien nicht durch einen geringeren Anteil der sich in S-Phase des Zellzyklus befindlichen Zellen hervorgerufen wurden.
Zellen mit einem Defekt im Signalweg der Homologen Rekombination sind häufig sensibel für quervernetzende Chemotherapeutika wie Cisplatin und MMC aber auch für PARP-Hemmer (16).
Wir untersuchten nun mittels Koloniebildungs-Tests die Sensibilität der 16 NSCLC Zelllinien gegenüber Cisplatin. Zwei Zelllinien (H1915 und H2087), bei denen sich eine herabgesetzte Fähigkeit zur Formierung von RAD51- Reparaturfoci ergeben hatte, reagierten hypersensibel auf die Behandlung (**Abb. 3**), während sich die beiden anderen Zelllinien (H1563 und H2126) mit defizitärer Bildung von Reparaturfoci resistent gegenüber Cisplatin erwiesen.

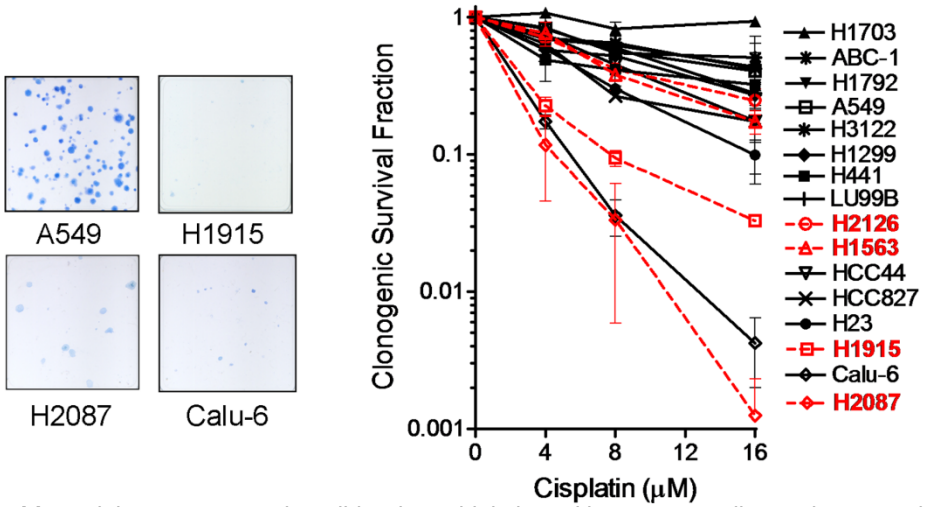

Abb.3: Links: Repräsentative Bilder der Fähigkeit zur klonogenen Zellvermehrung nach Behandlung von Cisplatin-resistenten A549 Zellen (200 Zellen ausgesät) und Cisplatin-sensiblen Zellen (H1915, H2087 und Calu6, jeweils 2000-5000 Zellen ausgesät) mit Cisplatin. Rechts: die klonogene Überlebensfraktion ist gegen eine steigende Cisplatinkonzentration aufgetragen. Die Datenpunkte und Kurven repräsentieren Mittelwerte mit Standardabweichung aus mind. 2 unabhängigen Experimenten. Zelllinien mit vermeintlichem RAD51-Defekt sind in Rot dargestellt.

Da jedoch zahlreiche andere Mechanismen existieren, anhand derer eine Zelle Resistenzen gegenüber Cisplatin entwickeln kann (17), wurde ebenfalls die Sensibilität auf MMC in den vier RAD51-defizienten Zelllinien untersucht. In allen vier Ziellinien zeigte sich ein reduziertes klonogenes Wachstum im Vergleich zu den RAD51-profizienten Zellreihen (**Abb.4**).

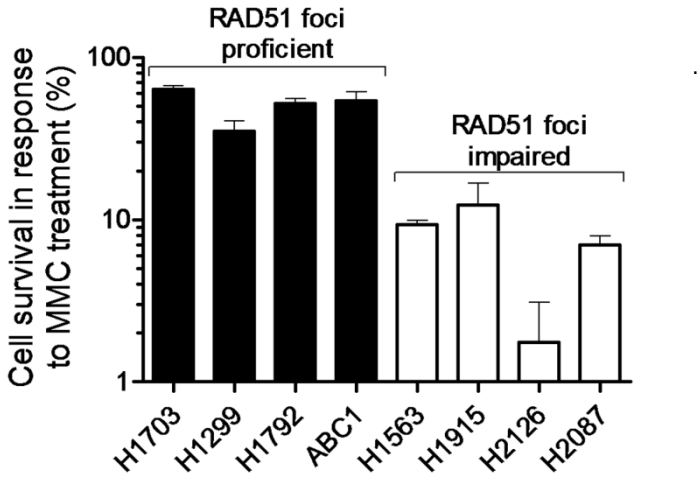

Abb.4: 8 NSCLC Zelllinien wurden 1 Std. mit 0,25mg/ml Mitomycin C (MMC) behandelt und deren anschließende Fähigkeit zu Koloniebildung gemessen

Erstaunlicherweise befand sich unter den Zelllinien, welche keine herabgesetzte RAD51 Aktivität aufwiesen, eine Tumorzellreihe (Calu-6), die ausgesprochen sensibel auf Cisplatin reagierte (**Abb.3**). Da sich der Defekt im Signalweg der Homologen Rekombination jedoch parallel oder stromabwärts der Aktivierung von RAD51 befinden könnte, untersuchten wir Calu-6 auf residuale γH2AX-Foci, einem Marker für DNA-Doppelstrangbrüche (18). Dabei zeigte sich nach 24 Std. eine erhöhte Anzahl von Doppelstrangbrüchen, was auf das Vorhandensein eines

Defektes der rekombinanten DNA-Reparaturprozesse dieser Tumorzelle hindeuten könnte (**Abb.5**).

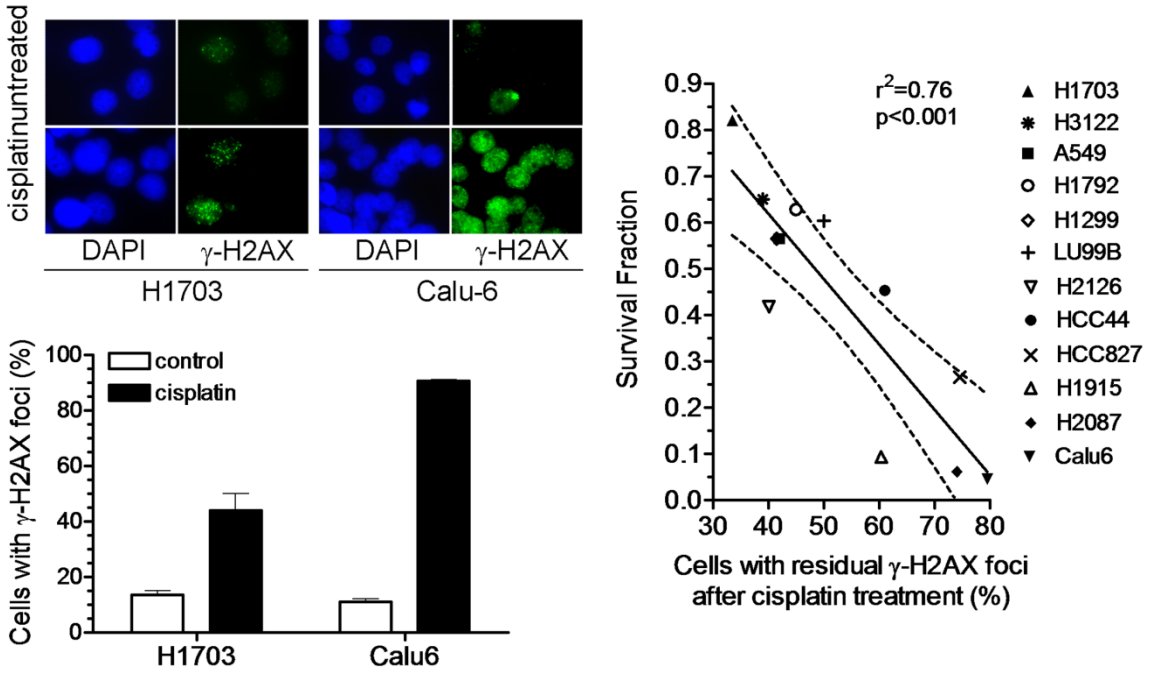

Abb.5: Links: Die beiden Abbildungen illustrieren die unterschiedlichen Formierung von γH2AX-Foci 24 Std. nach Behandlung von Cisplatin-resistenten H1703 und Cisplatin-sensiblen Calu6-Zellen mit 8µM Cisplatin. Rechts: Korrelation zwischen klonogenem Zellüberleben und residualen γH2AX-Foci 24 Std. nach Behandlung mit 8µM Cisplatin. Gepunktete Linien repräsentieren die 95%KI-Grenze der linearen Regressionslinie.

Wir erweiterten die Anwendung von γH2AX als Marker von DNA Doppelstrangbrüchen auf die anderen Zelllinien, und verglichen die Anzahl von residualen Foci nach 24 Std in den jeweiligen Zelllinien mit deren klonogenen Überlebensfähigkeit jeweils nach Behandlung mit Cisplatin. Dabei ergab sich für alle Zelllinien eine exzellente Korrelation($p<0,001$) zwischen nicht reparierten DNA-Doppelstrangbrüchen, gemessen an der Anzahl an residualen Foci, und der Sensibilität auf die Behandlung mit Cisplatin (**Abb.5**). Dies deutet darauf hin, dass sich sowohl RAD51 als auch γH2AX als Marker für funktionell relevante Defekte in rekombinanten DNA-Reparaturmechanismen, welche mit erhöhter Cisplatinsensibilität assoziiert sind, eignen.

Eine Zelllinie, die ebenfalls eine hohe Sensibilität gegenüber Cisplatin aufwies, ist H2087 (**Abb.3**), welche eine Mutation im Gen für die ATM-Kinase (ataxia-telangiectasia-mutated) beherbergt. Von ATM ist bekannt, dass es eine Rolle in der DNA Reparatur von durch Bestrahlung hervorgerufenen DNA Doppelstrangbrüchen durch nicht-homologes End-Joining und Homologe Rekombination spielt(19). Jedoch ist ATM noch nicht im Zusammenhang mit einer Cisplatinresistenz in Tumorzellen untersucht worden. Anhand von Untersuchungen in verschiedenen Zellsystemen konnten wir zeigen, dass ATM in die DNA Reparaturvorgänge nach Cisplatinbehandlung involviert ist und dass eine Mutation im Gen für ATM oder ein funktioneller Defekt der Kinase zu Hypersensibilität einer Tumorzelle gegenüber Cisplatin führt (**Abb.6**).

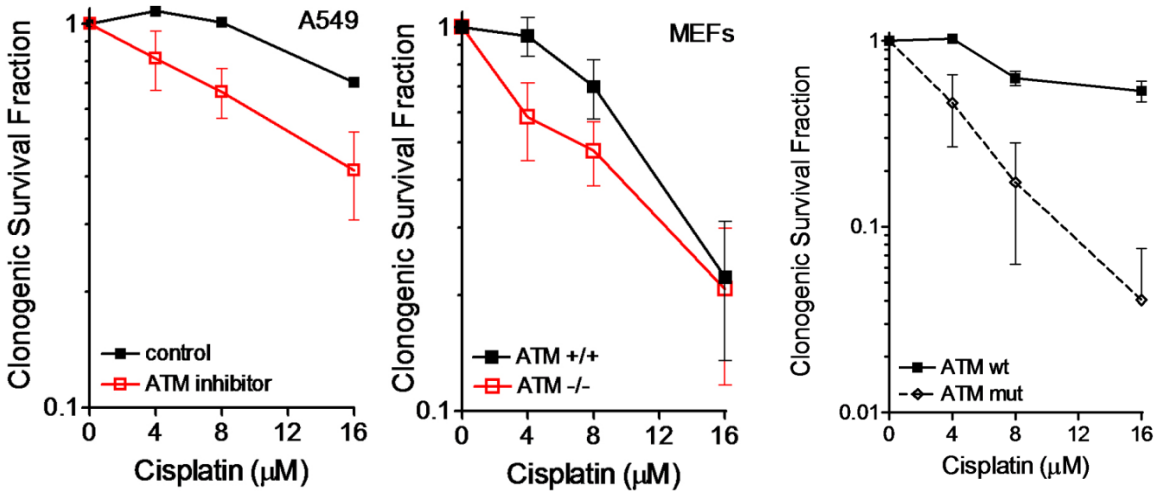

Abb.6: Links: unbehandelt (schwarz) und mit dem ATM-Inhibitor KU55933 vorbehandelte Zellen (20,μM, rot) wurden aufsteigenden Cisplatinkonzentrationen exponiert und klonogenes Wachstum wurde gemessen. Mitte: Klonogenes Wachstum nach Cisplatinbehandlung von mouse embryonic fibroblasts (MEFs) mit entsprechendem Genotyp. Rechts: Klonogenes Wachstum nach Cisplatinbehandlung von humanen Fibroblasten mit wildtyp (wt) und mutiertem (mut) ATM.

Die in der NSCLC-Zelllinie H2087 vorhandene Mutation in ATM bedingt eine herabgesetzte Phosphorylierung der Kinase an S1981. Die Kinetik der ATM Aktivierung glich der Kinetik der Induktion von Doppelstrangbrüchen (**Abb.7**). Phosphoryliertes ATM (p-ATM) konnte innerhalb von 6 Stunden nach Behandlung der Zellen mit Cisplatin detektiert werden und ko-lokalisierte im Zellkern mit DNA-Doppelstrangbrüchen (**Abb.8**).

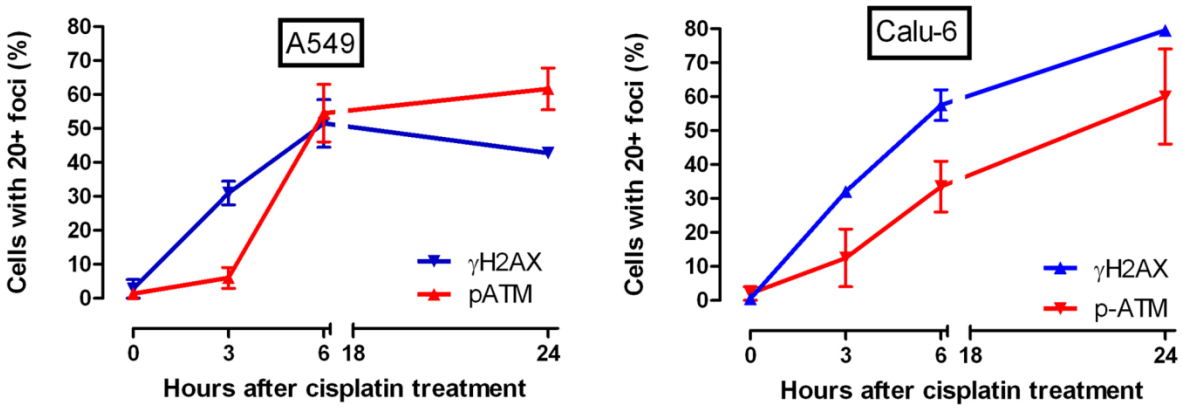

Abb.7: Links: Kinetik von γH2AX und p-ATM (S1981) Foci in A549 und Calu-6 Zellen nach Behandlung mit Cisplatin (1Std, 8μM)

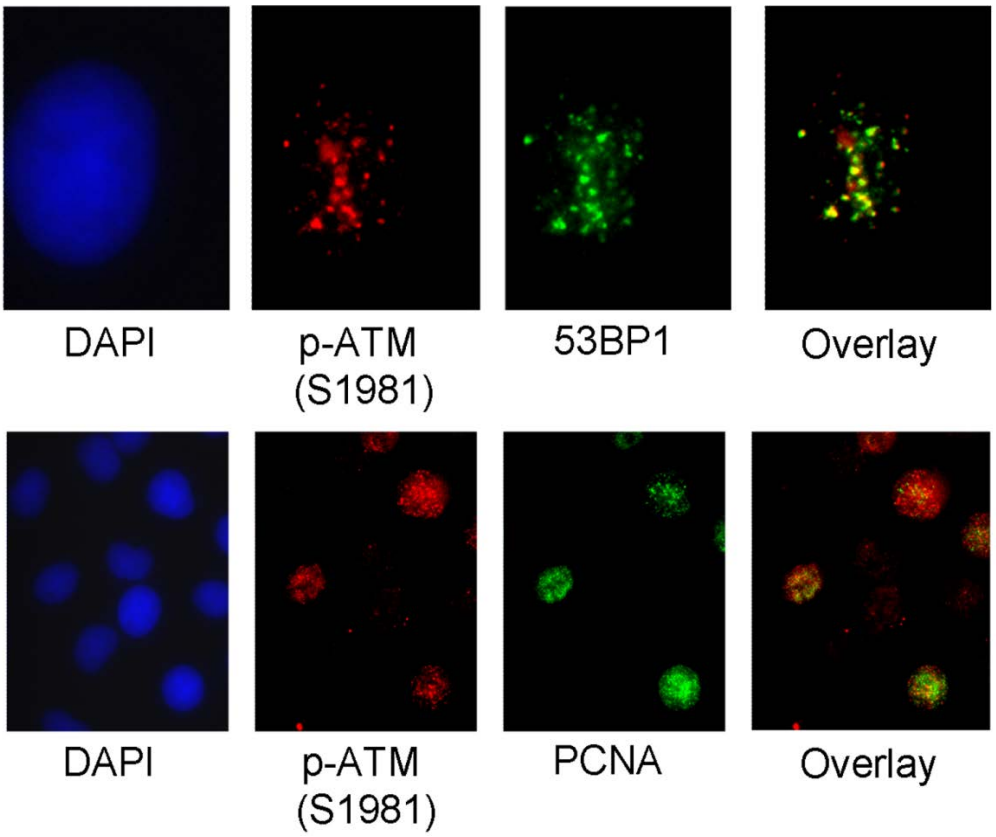

Abb.8: Repräsentative Aufnahmen von A549 Zellen, die eine Ko-Lokalisation von p-ATM-Foci mit dem DNA-Doppelstrangbruch-Marker 53BP1 oder dem S-Phase Marker PCNA 24 Std. nach Behandlung mit Cisplatin zeigen.

Um zu erforschen ob sich die aus den Zellversuchen gewonnenen Erkenntnisse auch auf vitales Tumorgewebe übertragen lassen, wurden 13 frisch entnommene Tumorexplantate ex vivo mit Cisplatin oder Bestrahlung behandelt und auf ihre Fähigkeit untersucht, RAD51 Reparaturfoci zu entwickeln, sowie DNA Doppelstrangbrüche zu reparieren (residuale γH2AX-Foci)(**Abb.9**). Dabei zeigte sich eine im Vergleich zu den Zelllinien deutlich herabgesetzte RAD51-Fociformation: durchschnittlich bildeten sich in lediglich 5,8% der Tumorzellen (95%KI, 3,0-8,7%) durch Cisplatin induzierte Reparaturfoci. Dieses Phänomen konnte durch einen im Vergleich zu den Zelllinien deutlich geringeren Anteil der sich in S-Phase des Zellzyklus befindlichen Zellen erklärt werden. In zwei der 13 Tumorgewebe (15%) wurde eine signifikant geringere Anzahl von Cisplatin-induzierten RAD51 Foci beobachtet (<1%der Zellen), sodass bei diesen Tumoren (L006 und L007) eine beeinträchtigte DNA-Reparatur durch Homologe Rekombination vermutet werden kann.
Anhand dieser Ergebnisse konnte zum ersten Mal gezeigt werden, dass sowohl durch Chemotherapeutika induzierte DNA-Schäden als auch die Intaktheit rekombinanter DNA-Reparaturwege in vitalem Tumorgewebe ex vivo anhand von Biomarkern beurteilt werden können, sodass sich Prognosen über die Wirksamkeit einer platinbasierten Chemotherapie abschätzen lassen.

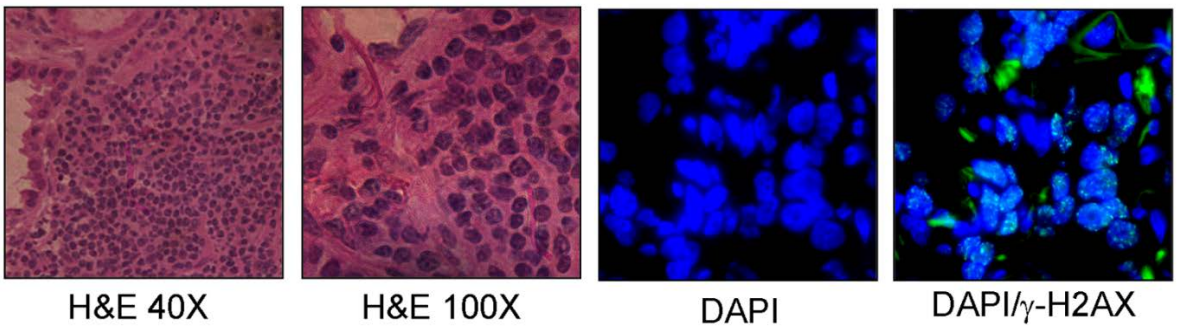

Abb.9: Eine Serie von tiefgefrorenen Schnellschnitten wurde mittels HE-Färbung auf vitales Tumorgewebe (1. und 2.Bild von links) sowie per Immunfluoreszenzmikroskopie auf DNA Doppelstrangbrüche hin untersucht (γH2AX-Foci, grün). Gegenfärbung der Zellkerne mit DAPI (blau).

Zusammenfassung

In der modernen Krebsforschung ist die Identifikation von Tumoren mit Defekten der DNA-Reparaturwege, insbesondere der Homologen Rekombination, bis heute eine große Herausforderung, vor allem wegen der Komplexität des zugrunde liegenden Signalwegnetzwerkes (10). Zudem ist unklar, ob Defekte in einzelnen Komponenten der Signalkaskade auch zu einer funktionellen Beeinträchtigung der DNA-Reparatur führen. Wachsendes Interesse gilt daher in jüngster Zeit der Untersuchung von Tumorzellen auf deren Fähigkeit zur Bildung subnukleärer Foci von zentralen Proteinen des DNA-Reparatursignalwegs.
Ein großer Vorteil dieses Ansatzes ist, dass man anhand eines „globalen Messinstruments“ die Funktionalität von DNA-Reparaturwegen überprüfen kann, ohne um die genaue Ursache des Defekts (z.B. genetische Mutationen, Veränderungen der Signaltransduktion) zu wissen, von denen bis heute noch viele Faktoren unbekannt sind.
Mit dieser Arbeit konnte ein Zusammenhang zwischen herabgesetzter RAD51-Fokusformation und Tumorsensibilität gegenüber Cisplatin oder MMC in nicht-kleinzelligen Bronchialkarzinomzellen gezeigt werden.
Dass außerdem eine starke Korrelation von Cisplatinsensibilität und residualen γH2AX-Foci gefunden wurde, spricht dafür, dass sich diese Marker oder eine Kombination aus beiden zum Einschätzen der Sensibilität auf eine platinbasierte Chemotherapie eignen.
Zudem konnte erstmalig ein Zusammenhang zwischen einem Defekt in ATM sowohl auf genetischer Ebene als auch in einer Funktion des Genprodukts und zellulärer Cisplatinsensibilität aufgezeigt werden. Da 7% der nicht-kleinzelligen Bronchialkarzinome eine Mutation in ATM aufweisen (20), könnte eine zelluläre Untersuchung der Funktionalität von ATM in Zukunft entscheidende Hinweise auf das Ansprechen einer platinbasierten Chemotherapie bei einem Teil der Patienten mit Lungenkrebs liefern.

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Erklärung des Eigenanteils:

Mein Anteil an der vorliegenden Arbeit besteht zunächst in der eigenständigen Planung und Durchführung der experimentellen Versuche in den Laboratorien der Radiation Oncology des Massachusetts General Hospital/Harvard Medical School. Die Auswertung der gewonnenen Ergebnisse in Form von Grafiken, Diagrammen und Bildern gehört ebenfalls zu meinem Anteil. Das Schreiben des Manuskriptes entstand in gemeinschaftlicher Arbeit der Erstautoren, sowie Henning Willers, dem Leiter des Labors und Letztautor, der für die Einreichung des Papers verantwortlich ist.
Nachfolgeversuche, wie das Übertragen der Zellversuche auf Gewebe wurden von Natalie Ferraiolo oder Liliana Gheorghiu durchgeführt. Die Rekrutierung von Patienten mit nicht-kleinzelligem Bronchialkarzinom, die chirurgische Entnahme von Tumoren, sowie das Anfärben in HE durch die Pathologie sind u.a von den verbleibenden Koautoren durchgeführt worden.

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Hamburg, 23. Januar 2013

Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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Unterschrift: .....