Evaluation of Pharmacodynamic Responses to Cancer Therapeutic Agents Using DNA Damage Markers

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Abstract

Purpose: We sought to examine the pharmacodynamic activation of the DNA damage response (DDR) pathway in tumors following anticancer treatment for confirmation of target engagement.

Experimental Design: We evaluated the time course and spatial activation of 3 protein biomarkers of DNA damage recognition and repair (γH2AX, pS343-Nbs1, and Rad51) simultaneously in a quantitative multiplex immunofluorescence assay (IFA) to assess DDR pathway activation in tumor tissues following exposure to DNA-damaging agents.

Results: Because of inherent biological variability, baseline DDR biomarker levels were evaluated in a colorectal cancer microarray to establish clinically relevant thresholds for pharmacodynamic activation. Xenograft-bearing mice and clinical colorectal tumor biopsies obtained from subjects exposed to DNA-damaging therapeutic regimens demonstrated marked intratumor heterogeneity in the timing and extent of DDR biomarker activation due, in part, to the cell-cycle dependency of DNA damage biomarker expression.

Conclusions: We have demonstrated the clinical utility of this DDR multiplex IFA in preclinical models and clinical specimens following exposure to multiple classes of cytotoxic agents, DNA repair protein inhibitors, and molecularly targeted agents, in both homologous recombination-proficient and -deficient contexts. Levels exceeding 4% nuclear area positive (NAP) γH2AX, 4% NAP pS343-Nbs1, and 5% cells with ≥5 Rad51 nuclear foci indicate a DDR activation response to treatment in human colorectal cancer tissue. Determination of effect-level cutoffs allows for robust interpretation of biomarkers with significant inter-patient and intratumor heterogeneity; simultaneous assessment of biomarkers induced at different phases of the DDR guards against the risk of false negatives due to an ill-timed biopsy.

Introduction

Therapeutic agents that induce cell death by directly damaging DNA remain mainstays of cancer treatment. As new therapeutic agents enter the clinical arena and novel combination therapy approaches are explored, there is a growing need to monitor DNA damage repair (DDR) directly in patient tumors to provide proof of mechanism for novel agents, to evaluate potential causes of cytotoxic potentiation, as well as to understand differences in drug responses for individual patients (1). Assays of primary and secondary pharmacodynamic effect are particularly suited for these applications, because they can provide direct evidence of target engagement, unlike downstream markers of drug effect such as cellular apoptosis, which may predict drug response but do not clearly indicate a mechanism of action. With the renewed interest in epigenetic and DNA-damaging agents for their ability to combine with immunotherapy, for example, it will be critical to know whether DNA damage occurred as predicted.

Establishing the pharmacodynamic response of DNA damage indicators, such as the biomarker of DNA double-stranded breaks (DSB) histone H2AX phosphorylated at Ser139 (γH2AX; refs. 2–5), in the preclinical setting can also guide the development of clinical drug administration and biomarker collection scheduling. Our experience, however, is that preclinical pharmacodynamic outcomes may not be fully recapitulated in clinical studies. Analysis of biopsies from patients with advanced solid tumors who received combination treatment with veliparib and irinotecan did not replicate the increase in γH2AX levels expected from preclinical modeling (6). In such cases, the discordance between preclinical and clinical observations could stem from numerous factors; notably, in most clinical trials only a single posttreatment tumor sampling is feasible. Hence, a difference of a few hours in collection time could substantively alter the likelihood of observing activation of certain biomarkers.
Translational Relevance

Nuclear proteins γH2AX, pS343-Nbs1, and Rad51 are critical components of the cellular DNA damage response (DDR). We demonstrate in this study that a panel of these three biomarkers can usefully assess the cellular pharmacodynamic response, needed for establishing mechanism of action and developing drug administration schedules, to multiple classes of DNA-damaging therapeutic agents. We also determined natural baseline levels and variation of these biomarkers in tumor specimens from 43 patients undergoing initial surgery for colorectal cancer, thereby establishing effective threshold levels of activation for each biomarker. Quantitation of the DDR using a multiplex immunofluorescence assay for γH2AX, pS343-Nbs1, and Rad51 permits simultaneous evaluation of multiple pharmacodynamic responses, which helps increase the likelihood of observing pharmacodynamic responses that can vary between individuals based on biopsy timing, drug and dose studied, and the genetic background of the patient.

Moreover, the induction of a given biomarker can be the result of DNA repair or apoptosis. Without using newer methods that distinguish these two outcomes, the interpretation of DDR, we incorporated two additional biomarkers into our validated immunofluorescence assay (IFA) for γH2AX (4, 6), Nbs1 was selected because of its contribution to the Mre11-Rad50-Nbs1 (MRN) complex (critical for early DNA damage recognition, processing, and signaling), ATM recruitment to damage sites, DSB repair, and cell-cycle checkpoint activation (8–11). The phosphorylation status of Nbs1 has been shown to dictate downstream repair choices between error-prone nonhomologous end joining (NHEJ) and error-free homologous recombination (HR) using homologous sister chromatids, in a cell-cycle-dependent manner (12). ATM-dependent phosphorylation of Nbs1 at S343 occurs in response to DSBs (13–15), making pS343-Nbs1 a valuable addition to our assay. Rad51, an essential component of the homologous recombination repair pathway, forms foci at DSB sites and can be quantified to measure DNA damage repair, and homologous recombination pathway status (16–20). Rad51 has also recently emerged as a critical component of DDR, with 10% FBS (Lonza) and 1% penicillin-streptomycin (Lonza).

Drug-treated animal models

A total of 229 human colon cancer, A375 human melanoma, A2780 ovarian cancer, A673 Ewing sarcoma, and HCT-116 colorectal carcinoma cell lines (ATCC) were grown in RPMI supplemented with 10% FBS (Lonza) and 1% penicillin-streptomycin (Lonza).

Materials and Methods

Cells and reagents

HCT-29 human colon cancer, A375 human melanoma, A2780 ovarian cancer, A673 Ewing sarcoma, and HCT-116 colorectal carcinoma cell lines (ATCC) were grown in RPMI supplemented with 10% FBS (Lonza) and 1% penicillin-streptomycin (Lonza).

Primary antibody validation and conjugation

All primary mAbs were purchased from commercial vendors: pS343-Nbs1 (rabbit mAb, Abcam), γH2AX (mouse mAb, Millipore), and Rad51 (rabbit mAb, Epitomics; mouse mAb, Novus Biologicals). Certificates of Analysis were provided for all antibodies. Antibodies were custom-conjugated to fluorescent dyes or hapten: anti-γH2AX-biotin (Millipore), anti-γH2AX to Alexa Fluor 790, anti-pS343-Nbs1 to Digoxygenin (DIG) and anti-Rad51 to Dinitrophenol (DNP; Molecular Probes, Inc.). New and previously qualified lots of antibodies were compared side-by-side. Validation details can be found in the Supplementary Methods and Supplementary Figs. S1 and S2, and in LoRusso and colleagues for pS343-Nbs1 (6). Cell cycle and apoptosis antibodies used were as follows: p21 Waf1/Cip1 (12D1), cyclin B1 (D5C10), p-histone H3-Ser 10 (rabbit mAb, Cell Signaling Technologies), cyclin B1-Alexa-647 [rabbit mAb, (EPRI7060) Abcam], geminin (mouse mAb, Abcam), and cleaved caspase-3 rabbit antibody (R&D Systems).

Drug-treated animal models

Athymic nu/nu mice were implanted with A375 (topotecan-responsive), A673, HCT-116, or A2780 cells as described previously (4, 25). A375 xenograft quadrants were collected from mice 4 hours after intraperitoneal administration of vehicle (sterile water) or 4.7 mg/kg topotecan. The MTD for topotecan administered to mice once daily for 5 consecutive days (QD×5) is 4.7 mg/kg/day (4, 26, 27). A673 xenograft quadrants were collected from mice following intraperitoneal administration of vehicle (saline) or 240 mg/kg gemcitabine at 4, 7, 12, 24, or 52 hours after 1 dose. HCT-116 xenografts were collected from mice following oral administration of vehicle (saline) or 5-fluorouracil (60 mg/kg) at 24 hours after the first dose on a 5 × Q2D schedule; or following intraperitoneal administration of vehicle (saline), 2 mg/kg 5-aza-T-dCyd (NSC 777586) or 0.75 mg/kg decitabine (NSC 127716) on treatment day 11 following 10 doses on the schedule QD×5, rest 2 days, QD×5, rest 9 days. A2780 xenografts were collected from mice following intraperitoneal administration of vehicle (saline) or 6 mg/kg cisplatin (NSC 119875) at 4, 7, or 24 hours after 1 dose. Samples were fixed in neutral-buffered formalin, and paraffin-embedded as described previously (4).

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In this report, we establish the natural range of expression of pS343-Nbs1, Rad51, and γH2AX in colorectal cancer clinical specimens to define response thresholds for the individual biomarkers that must be exceeded to indicate a pharmacodynamic drug response. Furthermore, we demonstrate broad applicability of our novel multiplex IFA to characterize DDR pathway activation following genotoxic stress in cellular and mouse cancer models with and without HR deficiencies, using formalin-fixed, paraffin-embedded (FFPE) specimens that have been rapidly cryopreserved at the point of collection with a proven procedure for stabilizing phosphoprotein biomarkers (24). These results establish assay fitness-for-purpose, which revealed robust biomarker responses occurring over a time frame suitable for clinical implementation with tumor core biopsies, that is, 4–6 hours after drug administration, and confirm the usefulness of multiplexing DDR markers to guard against the risk of false negatives. Notably, we describe for the first time the phosphorylation of Serine-343 on Nbs1 in response to several classes of DNA-damaging agents other than ionizing radiation and irrespective of p53 or BRCA1 status, and observed substantial inter- and intratumor heterogeneity in DDR biomarker activation, which we attribute to asynchronous progression through the cell cycle.
Human tumor biopsies

Pairs of 18-gauge core-needle tumor biopsies were collected from metastatic sites in patients with advanced disseminated disease refractory to prior therapy and enrolled in early-phase clinical trials conducted by the NCI’s Division of Cancer Treatment and Diagnosis (DCTD) and Center for Cancer Research at the NIH Clinical Center, Bethesda, MD. Biopsies were obtained before and 5 days after the initiation of treatment and analyzed for levels of DDR biomarkers γH2AX, pS343-Nbs1, and Rad51 using the quantitative IFA validated for use on FFPE human tissue as described below. All patients gave written informed consent for study participation. Study design and conduct complied with all applicable regulations, guidances, and local policies, and the study was approved by the NCI institutional review board. Biopsies were placed in prechilled cryogenic vials, snap-frozen within 2 minutes of collection, and stored at −80 °C. Biopsies were fixed and paraffin blocked together with a biomarker-positive control tissue for sectioning, following DCTD standard operating procedures (http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm).

Microarrays containing colonic or rectal adenocarcinoma and normal tissue adjacent to tumor were obtained from Indivumed GmbH. Tissue was derived from tumor resections, and ischemia times were less than 5 minutes until fixation in formalin or snap-freezing, with the exception of two tumor fragments that had ischemia times of 6 and 9 minutes. Formalin-fixed tissue cores were paraffin-embedded and supplied as blocks or tissue sections on slides.

Multiplex immunofluorescence staining

The method used for staining FFPE tissue sections was modified from our previously described Bond-max Autostainer Staining protocol (4); detailed Bond-max System (Leica Microsystems) methods can be obtained from the manufacturer. Two formats of γH2AX (clone JBW301) antibody were used. Either a biotin-conjugated γH2AX antibody, which was detected with the use of Streptavidin labeled with Alexa Fluor 660, or a γH2AX–Alexa Fluor 790 custom-conjugated antibody was used. Custom-conjugated pS343-Nbs1-DIG and Rad51-DNP antibodies were detected with the use of Alexa Fluor 546– or Alexa Fluor–467 IgG Fraction Monoclonal Mouse Anti-Digoxin (Jackson Immunoresearch) and Alexa Fluor 488 conjugated anti-DNIPhenyl-KLH Rabbit IgG Antibody fraction, (DNPI1488; Thermo Fisher Scientific). After staining with a cocktail of all three primary antibodies, followed by washing and staining with the secondary reporting antibodies (see Supplementary Methods for details), the slides were rinsed in 1 × PBS and blotted to remove excess liquid. Slides were cured overnight with Prolong Gold Antifade Reagent (Invitrogen) in the dark and imaged the following day. For long-term storage, slides were stored in the dark at −20 °C. For images used in assay development, slides were stained with individual antibodies using a method similar to the method above without the use of conjugated primary mAb. Confocal images (12-bit) were acquired at 20× using a Nikon A1 scan head on a Nikon 90i microscope at 0.24 μm/pixel, and 20× widefield images (14-bit) were acquired in the 790 nm channel using an Andor DU888 EMCDD camera at 0.65 μm/pixel.

Quantitative biomarker analysis

To restrict DDR-IFA analysis to primarily tumor cells, hematoxylin and eosin (H&E)-stained 5-μm sections from each biopsy or tissue microarray were annotated for areas of viable tumor by a pathologist, and this annotation was used to choose DDR-IFA image analysis fields on neighboring slides. Fields from at least two different slides and a minimum of 3,000 cells were required for each analysis. Definiens Architect XD Tissue Studio IF (Version 2.4.2.; Build 40013) software program was used for analysis of biomarker expression, coexpression (within the same cell), and nuclear enumeration. Definiens Tissue Studio software was used to quantify the changes in nuclear biomarker expression by two methods: nuclear area positive (NAP) analysis (defined by the Area of the Marker mask divided by the Area of the Nuclear Mask) for biomarkers displaying diffuse nuclear expression (γH2AX and pS343-Nbs1), or foci count per nucleus for biomarkers exhibiting distinct nuclear foci. See the Supplementary Methods and Supplementary Figs. S3–S5 for quantitation details. Quantitation was performed by cell enumeration based on a positive/negative cutoff for background fluorescence in each channel, for each image.

Statistical analysis

Descriptive statistics including mean, SD, SEM, and Student t test were conducted with Microsoft Excel. The significance level for the 95% confidence interval was set at α = 0.05 for a two-sided Student t test.

Results

Contrasting kinetics of pS343-Nbs1 and γH2AX responses to gemcitabine in vivo

While characterizing the time course of the DNA damage response in an Ewing's sarcoma xenograft model derived from A673 cells, a histology with known sensitivity to the cytotoxic cytidine analogue gemcitabine (28, 29), we noted a 24-hour delay in the induction of the DSB biomarker γH2AX in response to a single dose (at the mouse MTD—240 mg/kg) of gemcitabine (Fig. 1A and B). In contrast, robust induction of pS343-Nbs1 was detected within 2 hours of treatment, and the signal was sustained for at least 24 hours. The earlier induction of pS343-Nbs1 was concordant with its role in early DNA damage recognition, end processing, and signaling. As for γH2AX, its later activation is consistent with the mechanism of action of gemcitabine, which irreversibly inhibits ribonucleotide reductase leading to stalled replication forks and S-phase arrest, and eventually causes replication fork collapse and emergence of DSBs (30, 31). The complementarity of the time frames for pS343-Nbs1 and γH2AX induction in this model suggests a solution to the issue of selecting...
a single time point for clinical biopsy collection to assess the pharmacodynamic effects of drug treatment. Assessing biomarkers that are induced at different phases of the DNA damage response helps to ensure that a poorly timed biopsy will not result in a falsely negative pharmacodynamic signal. Therefore, we developed a multiplex quantitative IFA assay to assess the induction of γH2AX, pS343-Nbs1, and Rad51 in patient specimens to assist with pharmacodynamics-driven development of cancer therapeutics that damage DNA. Importantly, our characterization of pS343-Nbs1 established that it is induced in response to different classes of DNA-damaging agents including gemcitabine, topotecan, cisplatin, and ionizing radiation, irrespective of p53 or BRCA1 status (Supplementary Fig. S4A–S4C). As expected, pS343-Nbs1 was not activated by agents that do not directly induce DNA damage such as the proteasome inhibitor bortezomib (Supplementary Fig. S4D).

Biological baseline variability in human tumor specimens and determination of cutoff values for establishing a pharmacodynamic biomarker response

The DDR-IFA staining procedure for γH2AX, pS343-Nbs1, and Rad51 was developed to allow the use of both direct and indirect reporter methods, as described in Supplementary Materials and Methods. Baseline biological variability was established for each biomarker across 64 individual cores from 32 colorectal (CRC) tumor resections contained in a paraffin-embedded colorectal tissue microarray (TMA; Indivumed). The TMA also included 15 cores of normal tissue found adjacent to cancer. A comparison of the normal colorectal tissues and CRC tumor tissues showed that the DDR biomarkers had largely overlapping expression ranges, allowing us to pool the normal and tumor tissues for the purpose of establishing the biological baseline variability for colorectal tissue (Fig. 2A). The individual tissue cores analyzed had a minimum of 66 and maximum of 7,346 cells available for DDR-IFA analysis (median 1,657; mean 1,887) with cell counts in the normal tissue generally lower than those in the tumor tissue. At least 500 individual nuclei were analyzed for biomarker expression in greater than 95% of the cores, and ≥1,000 individual cells were analyzed in 88% of the cores.

To this dataset, we also added DDR-IFA baseline data from 11 baseline core-needle biopsies collected before initiation of protocol therapy from patients at the NCI Developmental Therapeutics Clinic with advanced-stage colon or colorectal cancer (Fig. 2B and C), for a total of 90 human colorectal tissue specimens. The tumor content of these biopsies was evaluated by pathologist review and a minimum of 4,000 individual tumor nuclei were quantitated across at least two nonadjacent sections to ensure a representative sampling of each tumor biopsy. More cells were analyzed for each core-needle biopsy specimen than for each specimen in the TMA because more tissue was available; however, we found the biomarker expression ranges from both groups were in good agreement. A 95% cut-off value was then determined for each biomarker from the pooled TMA (tumor and normal tissue) cores and core-needle biopsies so pharmacodynamic response in colorectal cancer could be defined as a biomarker value that exceeded biological baseline variability to allow small but significant biomarker inductions by drugs with unknown mechanisms to be measured. For this dataset of 90 individual colorectal tissues, 95% of the specimens had baseline values below 4% NAP for γH2AX, 4% NAP for pS343-Nbs1, and 5% of cells positive for Rad51 signal (measured as ≥5 foci/nucleus). These values did not change when tissues with less than 500 nuclei were excluded from the analysis.

We further assessed the variability of DDR biomarker baseline values in three independent patient-derived xenograft models of colon cancer origin (172845-121-T, CN0446-F447, and 3087
cases the baseline values were not significantly different from baseline heterogeneity, we use the expression cutoffs established above applied to models of this disease. The baseline values for all three biomarkers also fell below the 95% cut-off value established with the TMA (Fig. 2D). However, the DDR-IFA revealed DDR biomarker expression to be higher and more variable in 8 colon cancer cell lines compared with human tissue (Fig. 2E). These findings may not be surprising given the well-established differences between in vitro models and tumor tissue. The critical nature of understanding the baseline variability in interpreting clinical results is shown in a study of duplicate pretreatment biopsies analyzed for Rad51 (Fig. 2F). Ten biopsies from 5 patients were analyzed, with each of the duplicate biopsies per patient derived from the same tumor lesion. In all cases, the mean Rad51 signal measured across three sections fell below the effect-level cutoff of 5% Rad51 baseline expression, and in most cases the baseline values were not significantly different from each other. However, in patient 68, we did observe significantly different Rad51 expression between the duplicates, demonstrating that biological variability of baseline values alone could lead to misinterpretation of a change as a drug effect if an effect-level cutoff is not established. To avoid misinterpretations stemming from baseline heterogeneity, we use the expression cutoffs established here, rather than a comparison with baseline values, to more reliably define DDR biomarker activation for all colorectal cancers and derived tissues. Similar studies may be needed to define effect-level cutoffs in other cancer types.

Assessment of DDR activation in vivo and across drug classes

We assessed the newly established effect-level thresholds of the pharmacodynamic biomarker responses of relevant in vivo models in a time frame consistent with clinical sampling. We analyzed tumor quarters from mice bearing human tumor xenografts (4–5/group) pre- and posttreatment with a panel of approved and investigational anticancer agents using the DDR multiplex

Figure 2.
Baseline expression of DDR markers and establishment of response cut-off values. A, Baseline expression of selected DDR markers was demonstrated and quantified in a colorectal tissue array with 32 tumors (2 cores/case are included) and 15 normal colon tissues. At least 500 individual tumor nuclei were quantified in 95% of the TMA cores. B and C, Baseline marker quantitation in advanced stage colorectal cancers from patients enrolled in phase I clinical trials at NCI. Median expression and interquartile range is indicated for each marker. A minimum of 4,000 individual tumor nuclei were quantitated across at least two nonadjacent slides per biopsy specimen. D, Baseline expression in human colon adenocarcinoma patient-derived xenografts. At least 5,000 nuclei quantified per model. E, Baseline expression of selected DDR markers was demonstrated and quantified in 8 colorectal cancer cell lines. More than 1,000 individual nuclei were quantified for each cell line. F, Intra-lesion baseline Rad51 quantitation from patients with advanced stage cancers with two biopsies each collected from the same lesion. *, P < 0.05. The dashed line represents our empirically determined baseline value cutoff of 5% of cells ≥ 5 Rad51 foci per nucleus.

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Figure 3.
DDR multiplex is broadly applicable across classes of different genotoxic therapeutic agents in vivo. Representative immunofluorescence images showing the induction of DDR markers in a variety of xenograft models treated with different genotoxic agents: 60 mg/kg clofarabine 5xQ2D (A); 2 mg/kg S-aza-T-dCyd (AzaTdC) QD × 5 with 2-day rest (C); and 0.75 mg/kg decitabine (DAC) QD × 5 with 2-day rest in HCT-116 colon xenografts (E); single 6 mg/kg dose of cisplatin in A2780 ovarian (G); and 4.7 mg/kg topotecan QD × 5 in A375 melanoma xenografts (I). Resolution: 20×, confocal, 0.24 μm/pixel. Corresponding changes in DDR marker expression are shown in B, D, F, H, and J, and the timing of xenograft sampling is indicated on the x-axes. Although all three markers are significantly activated by treatment with the five agents, at the cellular level, DDR expression varies widely. Statistical significance for the difference in marker expression levels between vehicle-treated and drug-treated xenografts corresponds to *, P < 0.05; **, P < 0.01; ***, P < 0.001.
assay (Fig. 3). A minimum of 10,000 cells per treatment group were analyzed. A single 60 μg/kg dose of the purine nucleoside antimitabolite clofarabine, which slows the growth of HCT-116 xenografts but does not cause tumor regression (25), caused significant induction of all three biomarkers (24 hours after 1 dose; ***, P < 0.01 for Rad51 and pS343-Nbs1 and ***, P < 0.001 for γH2AX; Fig. 3A and B). Similarly, treatment with 2 mg/kg of the DNA-hypomethylating nucleoside analogue 5-aza-4′-thio-2′-deoxycytidine (5-aza-T-dCyd), a therapeutic dose [tumor volumes did not double during these two full cycles of treatment and the optimum treatment-to-control ratio of tumor volumes (T/C) was 12%], produced induction of all three biomarkers 24 hours after the tenth dose (***, P < 0.001 for Rad51 and pS343-Nbs1 and ***, P < 0.01 for γH2AX; Fig. 3C and D). In contrast, treatment with the nucleic acid synthesis inhibitor decitabine (Fig. 3E and F), which induces only tumor growth delay in HCT-116 xenografts (with a time-to-tumor-volume-doubling of 6 days and an optimum T/C of 44%), caused a modest increase in pS343-Nbs1 (***, P < 0.05) 24 hours after the tenth dose (0.75 mg/kg), which did not exceed the expected 4% NAP cutoff.

A2780 ovarian cancer xenografts treated with 6 mg/kg of the DNA cross-linking agent cisplatin, a dose that causes moderate tumor growth delay (32), showed significant induction of Rad51 and pS343-Nbs1 but not γH2AX within 7 hours of treatment; Rad51 expression further increased between 7 hours and 24 hours (***, P < 0.01). After 24 hours of treatment, all three biomarkers were significantly induced (Fig. 3G and H). Furthermore, analysis of A375 xenografts 4 hours after treatment with 4.7 mg/kg topotecan revealed a significant increase in the percentage of cells positive for Rad51 (***, P < 0.05) in addition to the significant induction of pS343-Nbs1 (***, P < 0.001) and γH2AX (***, P < 0.01; Fig. 3I and J). This biomarker response was associated with a dose of topotecan (4.7 mg/kg) that causes tumor regression in this model (4). γH2AX and pS343-Nbs1 expression was also examined at a lower dose of topotecan (0.5 mg/kg), which does not cause tumor regression but does elicit a moderate biomarker response and therefore serves as the minimum biologically effective dose that produces a change in the pharmacodynamic biomarkers that are distinguishable from the nontreated control group (Supplementary Fig. S6; ref. 4).

**DDR activation in the context of homologous recombination defects**

Although the same systematic evaluation of baseline DDR biomarker expression levels has not yet been carried out in ovarian and melanoma tissue arrays, the similar pattern of DDR biomarker activation observed in xenografts derived from HCT-116 colon, A2780 ovarian cancer, and A375 melanoma cell lines suggests that this DDR assay can be generally applied to the monitoring of pharmacodynamic responses to DNA-damaging agents regardless of tumor type. We have also observed the DDR assay to report target engagement in differing genetic backgrounds, such as severe homologous recombination defects (HRD). For example, in an MX-1 breast carcinoma xenograft model containing a frameshift in BRCA1 that results in a truncated protein at residue 999 and two BRCA2 nonsynonymous SNPs that have been described in cases of familial breast cancer (N289H and N991D; ref. 33), we observed similar baseline levels of the three DDR biomarkers and significant (***, P < 0.001) drug-induced Rad51 and pS343-Nbs1 inductions in response to irinotecan doses too low to inhibit xenograft growth (Supplementary Fig. S7A and S7B). In another case, an in vitro experiment with 1 μmol/L cisplatin in isogenic UWB1.289 ± BRCA1 ovarian carcinoma cells displayed similar expression of the DDR biomarkers in the two genetic backgrounds, with statistically significant (**, P < 0.05) induction of all three biomarkers after 24 hours of treatment (Supplementary Fig. S7C and S7D).

Further studies are required to determine whether DDR baseline variability is higher in HRD tumors compared with HR-competent tumors, but the results to date demonstrate that background HRDs do not preclude the induction of the three DDR-IFA biomarkers.

**Clinical readiness**

To establish the translational potential of measuring aspects of the DDR in the clinic, we applied the DDR multiplex panel to the analysis of 18-gauge tumor biopsies taken from liver metastases in 3 patients with colon cancer before and after 5 days of treatment with an investigational, DNA-damaging drug regimen (NCT01851369) consisting of the DNA-damaging agent temozolomide coadministered with the experimental DDR inhibitor TRC102 on days 1–5 of a 28-day cycle (Fig. 4). Despite the similarities in the diagnoses, site and timing of biopsies, drug regimen, lack of clinical response (all 3 patients came off study within the first two cycles of treatment), and negligible predose biomarker levels, each patient’s posttreatment biopsy displayed a distinct pattern of DDR biomarker induction. All 3 patients had ≤2% of cells positive for Rad51 at baseline; Rad51 levels were induced well above the effect-level cutoff of 5% (11%, 7%, and 17% for patients A, B, and C, respectively) after 5 days of treatment. However, the patients differed in induction of pS343-Nbs1 and γH2AX. After treatment, patient B displayed a 14% γH2AX signal, and patient C had 11% NAP pS343-Nbs1, both values above the established 4% NAP effect-level threshold. Overall, the induction of the DDR biomarkers was measurable using this multiplex biomarker panel, proving the feasibility of using this assay on clinical specimens.

**DDR heterogeneity and the role of cell cycle**

Across our in vivo experiments (Fig. 3) and in the three colon cancer patient specimens (Fig. 4), we observed heterogeneous patterns of DDR biomarker activation within individual cells and across tissues: a portion of cells expressed a single DDR biomarker, some expressed multiple biomarkers simultaneously, and some expressed none of the DDR biomarkers. Because DNA damage responses are intimately related to the cell cycle, we investigated whether the expression of DDR-IFA biomarkers could be tied to distinct cell-cycle phases. To this end, we measured the G1–S cell-cycle arrest biomarker p21 (34), the G2 cell-cycle arrest biomarker cyclin B1 (35), the mitosis biomarker phospho-S10-histone-H3 (pHH3), and the S-phase through early M-phase indicator geminin (36) in p53- and BRCA1/2-proficient A375 xenografts treated with 4.7 mg/kg topotecan at 4 hours, which caused significant induction of all three DDR biomarkers (Figs. 3B and 5A). Topotecan-treated tumors displayed a significant increase in nuclear p21 expression, indicative of p53-dependent, DNA damage–induced G1–S arrest (from 1.0% NAP at baseline to 12.9% NAP after treatment; ***, P < 0.001), along with a decrease in cells in G2 and mitosis compared with vehicle control as indicated by the decreased number of cells expressing cyclin casein kinase B1 (from 5.1% to 2.3%; **, P < 0.05) or chromatin-associated pHH3 (from 2.2% to 0.2%; ***, P < 0.001; Fig. 5A and B), respectively. No
change was seen in geminin expression, likely because IFA thresholding was set to capture all geminin-expressing cells, irrespective of signal intensity (Fig. 5B).

Colocalization data for the cell-cycle biomarkers and DDR-IFA biomarkers showed that the vast majority of pS343-Nbs1 and Rad51 colocalized with cyclin B1 and geminin but not with p21 (Fig. 5C, 3rd and 4th row, respectively), clearly indicating these biomarkers are activated during S and G2 phases and not at G1 arrest. The role of Rad51 in homologous recombination is well known, and it has also more recently been identified as an important component of replication fork reversal, associated with chemotherapy resistance (21–23); its expression during S phase and G2 is expected and agrees with in vitro experiments (Supplementary Fig. S8). Interestingly, the lack of colocalization between pS343-Nbs1 and p21 suggests that pS343-Nbs1 does not play a significant role in G1 arrest, but rather is active in DNA damage repair mechanisms in the S and G2 phases of the cell cycle, with cyclin B1 coexpressed in 8.7% of pS343-Nbs1–expressing cells, and geminin coexpressed in 95% of pS343-Nbs1–expressing cells. In contrast, γH2AX did not markedly colocalize with any of the cell-cycle biomarkers (Fig. 5C, 2nd row), but approximately 20% of the γH2AX-positive cells also displayed cleaved caspase-3 blebs, a biomarker of apoptosis (Fig. 5A; ref. 37). It is well established that γH2AX is a biomarker of both DNA damage and apoptosis and that colocalization of γH2AX with cytoplasmic cleaved caspase-3–positive blebbing identifies the apoptotic cells (37–39). The γH2AX-positive cells seen in these samples also display visible chromatin condensation and fragmentation, consistent with them being apoptotic cells. We summarize the heterogeneous DDR-IFA expression pattern observed in vivo in A375 xenografts treated with topotecan in Fig. 5D.

Discussion
Pharmacodynamic biomarkers are critical in early drug development to ensure that a drug's mechanism of action can be understood and can provide evidence of target engagement when developing administration schedules and rational combinations of drug types. Pharmacodynamic biomarkers of DNA damage response are somewhat unusual in that they could either be indicative of cells overwhelmed by DNA damage or actively involved in repairing DNA damage following exposure to a DNA-damaging agent; therefore, while the detection of these pharmacodynamic biomarkers provides definitive evidence of drug activity and target engagement, they may not be predictive of overall drug response. In the studies described here, we have
demonstrated the importance of using pharmacodynamic biomarkers to understand the variability in the activation of the DNA damage response both with respect to specific drug classes and the timing of individual DNA repair events following drug treatment. The multiplex approach enables measurement of several analytes in a single clinical specimen, maximizing the amount of information from small core-needle biopsies. Multiplexing also reduces the possibility of missing a pharmacodynamic response due to specimen timing, dose schedule, or genetic alterations in the tumor (compared with a single biopsy readout). For instance, the lack of modulation at a given time point in a single analyte assay could be wrongly interpreted as either no drug effect or a genetic defect that prevents modulation of the target, a risk that can be circumvented by multiplexing (40). The importance of this approach is illustrated by the treatment of A673 xenografts with gemcitabine (Fig. 1), in which measurable γH2AX levels were found at a much later time point than induction of pS343-Nbs1. Because clinical biopsies are usually collected at a single time point after drug administration, the use of a multiplex assay increases the probability of detecting a reliable pharmacodynamic biomarker signal, which could be used to refine tumor-sampling times and assessment of drug mechanism of action. Although the three pharmacodynamic biomarkers included in our multiplex (γH2AX, pS343-Nbs1, and Rad51) are most commonly associated with the sensing and repair of DSBs, Rad51 also provides a readout of replication fork stalling as a result of single-strand DNA breaks (21–23), making the DDR-IFA useful for interpreting whether prolonged replication fork stalling or single-strand break induction results in induction of DSBs in particular genetic backgrounds.

We also performed an extensive study of baseline levels of each DDR biomarker across 90 colon/colorectal human tissue specimens, 11 colorectal cancer core-needle biopsies, 3 patient-derived xenografts, and 8 colon cancer cell lines. These evaluations examined the biological range of DDR biomarker expression and determined cut-off values that must be exceeded to conclude that a pharmacodynamic response has occurred (Fig. 2). These efforts revealed small but significant variations in the baseline expression of the biomarkers, not only between specimens from patients with the same histology, but also between individual needle biopsies from the same lesion (Fig. 2F). In the latter example, the lack of modulation at a given time point in a single analyte assay could be wrongly interpreted as either no drug effect or a genetic defect that prevents modulation of the target, a risk that can be circumvented by multiplexing (40). The importance of this approach is illustrated by the treatment of A673 xenografts with gemcitabine (Fig. 1), in which measurable γH2AX levels were found at a much later time point than induction of pS343-Nbs1. Because clinical biopsies are usually collected at a single time point after drug administration, the use of a multiplex assay increases the probability of detecting a reliable pharmacodynamic biomarker signal, which could be used to refine tumor-sampling times and assessment of drug mechanism of action. Although the three pharmacodynamic biomarkers included in our multiplex (γH2AX, pS343-Nbs1, and Rad51) are most commonly associated with the sensing and repair of DSBs, Rad51 also provides a readout of replication fork stalling as a result of single-strand DNA breaks (21–23), making the DDR-IFA useful for interpreting whether prolonged replication fork stalling or single-strand break induction results in induction of DSBs in particular genetic backgrounds.
also apparent with pS343-Nbs1 and γH2AX based on the differences in paired tumor tissues (Fig. 2A). Differences in biomarker baseline values are the major limitation to reporting percent increases in pharmacodynamic biomarker values in human specimens and are often underappreciated. Our efforts to evaluate baseline expression of all three DDR biomarkers in colorectal tissue facilitated the determination of specific cut-off values below which 95% of baseline measurements fall; we could then confidently distinguish a small drug response from normal baseline variability in this tissue type. This approach is superior to the current method of reporting only a proportional increase, which may erroneously identify small increases from a very low baseline level as a meaningful pharmacodynamic response. Unfortunately, this approach is not yet standard because it requires access to high-quality tissue from the tumor type in question and resources to quantify thousands of high-content images of human tissues. However, the concordance of DDR biomarker activation patterns between the histologies that we investigated—colorectal and ovarian cancer, and melanoma—add confidence to the broader applicability of colorectal TMA–derived baseline levels (Fig. 3). In the future, we will extend the evaluation of Rad51, pS343-Nbs1, and γH2AX baseline expression levels to additional histologies.

Through extensive preclinical modeling using empirically determined cut-off values, we demonstrated biomarker responses to multiple DNA-damaging agents (Fig. 3; Supplementary Fig. S5). These experiments provided critical data required to determine whether each assay had sufficient dynamic range and was fit for the purpose of measuring pharmacodynamic drug responses affecting the DDR pathway in vivo. However, preclinical xenograft studies are more easily interpreted than clinical studies due to the ability to compare drug- to vehicle-treated groups in statistically significant samples of animals with homogeneous genetic backgrounds. In clinical specimens, which generally involve smaller samples and greater heterogeneity than preclinical models, the use of multiple biomarkers for the same pathway increases the chance of observing a pharmacodynamic signal and provides more flexibility in interpreting the pharmacodynamic outcome. We have illustrated this point in Fig. 4, in which we demonstrate significantly different DDR biomarker activation patterns in three clinical specimens from patients with colorectal cancer receiving the same DNA-damaging drug treatment, biopsied at the same time following therapy. Although the drug treatment increased Rad51 levels in all three patient specimens, the γH2AX and pS343-Nbs1 drug responses varied, underscoring the strength of the multiplex approach.

It is known that DSB repair is cell-cycle dependent and occurs at the earliest stage following a cytotoxic injury (34, 41–43). This is important for the interpretation of our DDR biomarker determinations and provides insight into the mechanisms of action of the investigational agents the biomarkers are used to evaluate. Because our IFA multiplex technique allowed the visualization and localization of the biomarker signal across a large tumor cell population in the tissue sampled, without tissue extraction (44), we could identify distinct cellular subpopulations within tumors based on biomarker expression patterns, reflecting the relationship of the DDR response to cell-cycle phases. Our data demonstrate the cell-cycle–dependent expression of DDR markers in response to topotecan treatment (Fig. 5) and may help to explain heterogeneous responses to this and other therapeutic agents that produce DNA damage. Specifically, we noted that cells arrested at the G1–S checkpoint (as indicated by p21 expression) did not substantially colocalize with any of the three DDR biomarkers quantified in our assay, and appeared mainly as DAPI-only–stained nuclei in DDR-IFA–staining images (Fig. 5A and C). Lack of DDR marker expression in this population may reflect the protective effect of the G1–S checkpoint, preventing progression into S phase and accumulation of DNA damage. Prolonged G1–S arrest may lead to a p53/p21 pathway–induced apoptosis, consistent with our observation of an increase in γH2AX–expressing cells. Coexpression analysis of DDR and cell-cycle biomarkers in future studies may allow for greater understanding of drug activity and biomarker effect within a cell-cycle context and may be particularly informative for the development of drug combinations in which specific cell-cycle phases are targeted.

In summary, we have found that multiple markers of DDR can be evaluated concomitantly in vivo following treatment with a variety of different cancer therapeutic agents that damage DNA, and that the variability of the baseline expression of these DDR biomarkers in human colon cancers, although not inconsequential, permits the clinical quantification of the effects of multiple classes of DNA-damaging oncologic drugs on the DDR pathway. Furthermore, we suggest that the heterogeneous nature of the response to these drugs in the clinic may, in part, be due to cell-to-cell, cycle-dependent variations in the capacity of the tumor to repair DNA.

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

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