Cancer Therapy: Clinical

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Monitoring Drug-Induced γH2AX as a Pharmacodynamic Biomarker in Individual Circulating Tumor Cells

Lihua H. Wang[1], Thomas D. Pfister[1], Ralph E. Parchment[1], Shivaani Kummar[3], Larry Rubinstein[5], Yvonne A. Evrard[2], Martin E. Gutierrez[3], Anthony J. Murgo[6], Joseph E. Tomaszewski[6], James H. Doroshow[3,4,6], and Robert J. Kinders[1]

Abstract

Purpose: Circulating tumor cells (CTC) in peripheral blood of patients potentially represent a fraction of solid tumor cells available for more frequent pharmacodynamic assessment of drug action than is possible using tumor biopsy. However, currently available CTC assays are limited to cell membrane antigens. Here, we describe an assay that directly examines changes in levels of the nuclear DNA damage marker γH2AX in individual CTCs of patients treated with chemotherapeutic agents.

Experimental Design: An Alexa Fluor 488–conjugated monoclonal γH2AX antibody and epithelial cancer cell lines treated with topotecan and spiked into whole blood were used to measure DNA damage–dependent nuclear γH2AX signals in individual CTCs. Time-course changes in both CTC number and γH2AX levels in CTCs were also evaluated in blood samples from patients undergoing treatment.

Results: The percentage of γH2AX-positive CTCs increased in a concentration-dependent manner in cells treated with therapeutically relevant concentrations of topotecan *ex vivo*. In samples from five patients, percent γH2AX-positive cells increased post-treatment from a mean of 2% at baseline (range, 0-6%) to a mean of 38% (range, 22-64%) after a single day of drug administration; this increase was irrespective of increases or decreases in the total CTC count.

Conclusions: These data show promise for monitoring dynamic changes in nuclear biomarkers in CTCs (in addition to CTC count) for rapidly assessing drug activity in clinical trials of molecularly targeted anticancer therapeutics as well as for translational research. *Clin Cancer Res; 16(3); 1073–84.* ©*2010 AACR.*

In early drug development clinical studies, the ability to assess the effect of chemotherapeutic agents on tumor cells during the course of treatment can allow an immediate determination of drug effect on its putative target. The primary means of assessing anticancer drug efficacy is tumor shrinkage, which often requires multiple follow-up visits to observe an effect that may not be achievable at all doses tested in a phase I trial or in single-dose regimens ("phase 0" trials). Pharmacodynamic effect can also be determined through examination of biopsied tumor tissue (1). However, biopsies in a clinical trial cannot be done frequently enough to describe the time course and peak of pharmacodynamic responses to the drug being tested. In addition, inclusion of frequent biopsies in a clinical trial can raise ethical concerns and limit patient recruitment (2). Faster and less invasive methods for measuring patient tumor response during drug treatment and identification of biomarkers to monitor drug effect on-target can streamline the clinical trial process by allowing treatments to be monitored and tailored to the patient based on tumor cell response.

Circulating tumor cells (CTC) are found at low levels in the peripheral blood of patients with both metastatic and primary tumors. Presence of CTCs in the bloodstream is considered predictive of disease progression in breast cancer (3–7). A multicenter study that monitored 430 patients with metastatic colorectal cancer also determined that the number of CTCs before and during treatment was an independent indicator of both progression-free and overall survival (8). In addition to assaying total number of CTCs as an indicator of disease state, assessment of biomarkers in CTCs has also been used with the specific purpose of characterizing the tumor type better to provide more tailored cancer therapy (9–12).

There are several ways to detect CTCs. The CellSearch system (Veridex) is an automated cell isolation system that has been approved by the Food and Drug Administration

Authors' Affiliations: [1]Laboratory of Human Toxicology and Pharmacology, [2]Applied and Developmental Research Support Directorate, Science Applications International Corporation-Frederick, Inc., National Cancer Institute-Frederick, Frederick, Maryland and [3]Medical Oncology Branch and [4]Laboratory of Molecular Pharmacology, Center for Cancer Research; [5]Biometric Research Branch, [6]Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Maryland

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Corresponding Author: Robert J. Kinders, Laboratory of Human Toxicology and Pharmacology, National Cancer Institute-Frederick, Building 315, Room 6, Frederick, MD 21702. Phone: 301-864-6410; Fax: 301-846-6536; E-mail: kindersr@mail.nih.gov.

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Translational Relevance

We have developed a novel quantitative assay for the detection of the nuclear DNA damage marker γH2AX in circulating tumor cells (CTC). In contrast to pharmacodynamic assessment in tumor biopsies, measuring nuclear biomarkers in CTCs allows more frequent monitoring throughout treatment via noninvasive sampling. Here, we describe the assessment of this pharmacodynamic assay in tumor cell lines and patient samples; this assay is now being used for exploratory correlative studies in clinical trials. Results from an initial set of patient data suggest that γH2AX levels in CTCs may be a more sensitive marker for assessing drug effects in patients than total CTC counts alone. This pharmacodynamic biomarker and assay therefore have the potential to inform the development of new anticancer agents and enable longitudinal monitoring of target response.

for the enumeration of CTCs as a prognostic indicator in patients with breast, colon, and prostate cancers (13–15). CTC isolation has also been used successfully to screen for tumor cell-surface biomarkers, such as HER2, insulin-like growth factor, and epidermal growth factor receptor, and to determine some genotypic and phenotypic traits of CTCs in patients (9, 16–21).

Phosphorylated H2AX (γH2AX) is a marker of DNA double-strand break (DSB) damage and has been proposed as an effective pharmacodynamic biomarker following treatment with topoisomerase I inhibitors, including topotecan (22–24). Following DSB damage, the ataxia telangiectasia mutated protein kinase becomes activated and initiates a signal transduction pathway mobilizing DNA damage repair proteins; this cascade of events is required to maintain genome integrity (25). Phosphorylation of H2AX at Ser^{139} by ataxia telangiectasia mutated is one of the first steps in recruiting DNA repair complexes to the site of DSBs (26). In cells treated with DNA-damaging agents, γH2AX foci appear in the nucleus within minutes in a dose-dependent manner, and γH2AX loss or dephosphorylation correlates time-wise with DNA repair (27). With continuing exposure to biologically effective drug doses, DSBs increase with time until the entire nucleus appears γH2AX-positive and nuclear disintegration is observed (28).

Ionizing and UV radiation and chemotherapeutic agents such as cyclophosphamide, temozolomide, topotecan, and camptothecin induce DNA damage, which can trigger H2AX phosphorylation and subsequent DNA repair (22, 23, 29–32). Chemotherapeutic agents such as topotecan and camptothecin derivatives bind to topoisomerase I, trapping a DNA-bound complex; the trapped topoisomerase I complex then generates DNA damage at replication forks and sites of transcription (33). This damage is converted to a DSB and results in ataxia telangiectasia mutated activation and accumulation of γH2AX (34). Several studies have concluded that induction of γH2AX foci can serve as a biomarker for DNA damage from cytotoxic agents (28, 35–39).

Here, we present data showing the development of a novel method to monitor nuclear γH2AX levels in patient CTCs following chemotherapeutic treatment. This approach allows the monitoring of pharmacodynamic effects of anticancer therapies over the course of treatment via minimally invasive methods. The method measures drug response as percent γH2AX-positive CTCs from the same cells reported in the total CTC count.

Materials and Methods

Patient enrollment. Blood was drawn from patients at the Center for Cancer Research, National Cancer Institute, NIH. These patients were enrolled in phase I clinical trials of investigational agents and had a variety of advanced malignancies. All patients had disease refractory to multiple prior chemotherapy regimens. Blood samples for spiking were drawn from healthy donors at the National Cancer Institute-Frederick. All patients and healthy donors gave their informed consent for study inclusion and were enrolled on institutional review board–approved protocols.

Cell lines and media. The epithelial tumor cell lines MCF7 (human breast adenocarcinoma), PC-3 (human prostate adenocarcinoma), HT-29 (human colorectal adenocarcinoma), and SKOV-3 (human ovarian adenocarcinoma) were obtained from the American Type Culture Collection. Cell culture medium, EMEM or RPMI 1640 (American Type Culture Collection), was supplemented with 10% FCS (American Type Culture Collection) and gentamicin sulfate (Biowhittaker). Cells were counted using the Cellometer Automatic Cell Counter (Nexcelom Bioscience).

Flow cytometric analysis. Cells were incubated with 10 μg/mL anti-phospho-γH2AX (Ser^{139}; clone JBW301; Millipore) conjugated to Alexa Fluor 488 (γH2AX-AF488; Millipore) for 30 min at 4°C. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data were acquired from 10,000 gated events, and staining was compared with the isotype control antibody (IgG; BD Biosciences).

Immunomagnetic enrichment and immunofluorescence analysis by the CellSearch system. The CellSearch CTC Epithelial kit (Veridex) and Control kit (lot no. A767; Veridex) were used per the manufacturer's protocol. Control samples, tumor cell line–spiked blood samples, and patient samples were processed in the CellTracks AutoPrep system (Veridex) and analyzed by the CellTracks Analyzer II (Veridex).

Blood collection. Blood from both healthy donors and patients was collected and processed according to the CellSearch manufacturer's instructions. Approximately 10 mL of whole blood were collected by venipuncture from each patient into a CellSave Preservative Tube

(Veridex). Blood samples were maintained at room temperature for a maximum of 96 h before processing.

Cell line drug treatment, fixation, permeabilization, and spiking protocols. Topotecan (GlaxoSmithKline), temozolomide (Schering), or rapamycin (Wyeth) was added to cultured cell lines at 80% to 90% confluence and incubated for 2 h at 37°C in 5% CO_2. Cell lines were fixed and permeabilized with the one-step BD Cytofix/Cytoperm Fixation/Permeabilization Solution kit (BD Biosciences). A 50 μL volume containing 600 ± 50 fixed and permeabilized cells was added to 7.5 mL whole blood from healthy donors and processed on the CellSearch system.

Online sample processing for CTC detection. After blood collection, 7.5 mL whole blood was used per patient, and all processing steps were done automatically by the CellTracks AutoPrep system, including plasma removal, cell resuspension, reagent addition, mixing, incubation, and aspiration steps. Blood samples were processed following the manufacturer's protocol.

Online γH2AX-AF488 or HER2-FITC antibody processing. For online staining of prefixed and permeabilized cell lines with γH2AX-AF488 or HER2-FITC (CellSearch HER2/neu Tumor Phenotyping Reagent; Veridex) antibodies, cells were processed using the online CTC detection protocol in the CellTracks AutoPrep system following the manufacturer's protocol. γH2AX-AF488 (10 μg/mL) or HER2-FITC (manufacturer's protocol) antibodies were added and processed simultaneously with the CK-PE and CD45-APC antibodies. Samples were then magnetically aligned in a cartridge within the MagNest cassette and transferred to the CellTracks Analyzer II for sample evaluation (19).

Sample evaluation. Samples were analyzed by the CellTracks Analyzer II and captured images that contained objects fulfilling the predetermined criteria for epithelial cells [CK-PE positive; 4′,6-diamidino-2-phenylindole (DAPI) positive; CD45-APC negative] were exported to a gallery format. In addition, checkboxes adjacent to images in the gallery format indicate cells that meet these predetermined criteria. Images exported to the gallery were then examined independently by two operators to determine the final classification of cells.

CTC images were classified based on morphology (epithelial-like with a 4 μm minimum diameter) and immunohistochemical staining consistent with that of an epithelial cell. γH2AX-AF488 or HER2-FITC antibody-labeled CTCs were classified as γH2AX-positive CTCs or HER2-positive CTCs, respectively.

Precision and accuracy of control CTC recovery. Recovery experiments were conducted with the CellSearch CTC Control kit. Control samples used to mimic patient sample handling were processed according to the manufacturer's protocol to verify initial CTC recovery. Samples were then manually washed three times with the wash buffer from the CellSearch Epithelial Cell kit, still within the CellSearch-supplied cartridge inside the MagNest cassette. The washed CTCs were manually resuspended in 450 μL wash buffer and aligned magnetically within the MagNest cassette. The post-wash recovered CTCs were rescanned by the CellTracks Analyzer II to determine the final count of mock antibody-stained CTCs (post-wash count). The interday and intraday measurements were done by the same operator under the same conditions. The reproducibility of CTC analysis by different operators was measured on duplicate samples processed under similar conditions. Procedural control samples were processed following the manufacturer's protocol.

Precision and accuracy of γH2AX-positive CTC recovery. The following procedure was used to estimate the precision and accuracy of the detection of γH2AX-positive CTCs. Blood from healthy donors was collected into CellSave Preservative Tubes and dispensed into five aliquots of 7.5 mL. The PC-3 prostate tumor cell line was treated with 0, 0.01, 0.1, 1.0, and 10 μmol/L topotecan for 2 h at 37°C. A 50 μL volume containing 600 ± 50 topotecan-treated PC-3 cells was added to each tube of blood. The five tubes were then processed and analyzed by a single operator following the offline antibody staining protocol and run in the CellSearch system using the CTC control program to quantify the final γH2AX-positive CTC recovery. This entire procedure was repeated three times in the same day for the intraday variation or on three different days for the interday variation.

Offline antibody staining protocol of clinical samples. Because the online CellSearch system staining protocol is optimized for cell surface markers, staining of clinical samples for γH2AX was done offline following CTC enrichment. After online sample processing for CTC detection and sample evaluation to determine total number of CTCs, the isolated CTCs were fixed and permeabilized with the one-step BD Cytofix/Cytoperm Fixation/Permeabilization Solution kit (BD Biosciences). Cells were then incubated with 5 μg/mL γH2AX-AF488 for 20 min at room temperature. The antibody-labeled CTCs were then manually washed three times, still within the CellSearch-supplied cartridge inside the MagNest cassette, using the wash buffer from the CellSearch Epithelial Cell kit. The washed CTCs were manually resuspended in 450 μL wash buffer and aligned magnetically within the MagNest cassette. The γH2AX-labeled CTCs were rescanned by the CellTracks Analyzer II to determine the final number of γH2AX-positive CTCs.

γH2AX fluorescent microscopy. γH2AX image capture was conducted using Leica 5000 DM with a ×20 Plan Apo Ph2 (numerical aperture 0.75) objective and a Retiga 2000R monochrome CCD camera (QImaging). Individual nuclear foci cannot be shown by these methods due to the low magnification on the Veridex instrument (×20). Digital amplification will degrade image quality before the ×1,000 magnification needed to visualize foci is reached.

Images were acquired by pseudo-random selection. Manual exposure corrections were made based on the intensity of the DAPI signal for each field. Acquisition of γH2AX signal was normalized by adjusting camera exposure and excitation time such that the mean intensity of background autofluorescence was nulled out, and these exposure settings were used for all fields examined in the

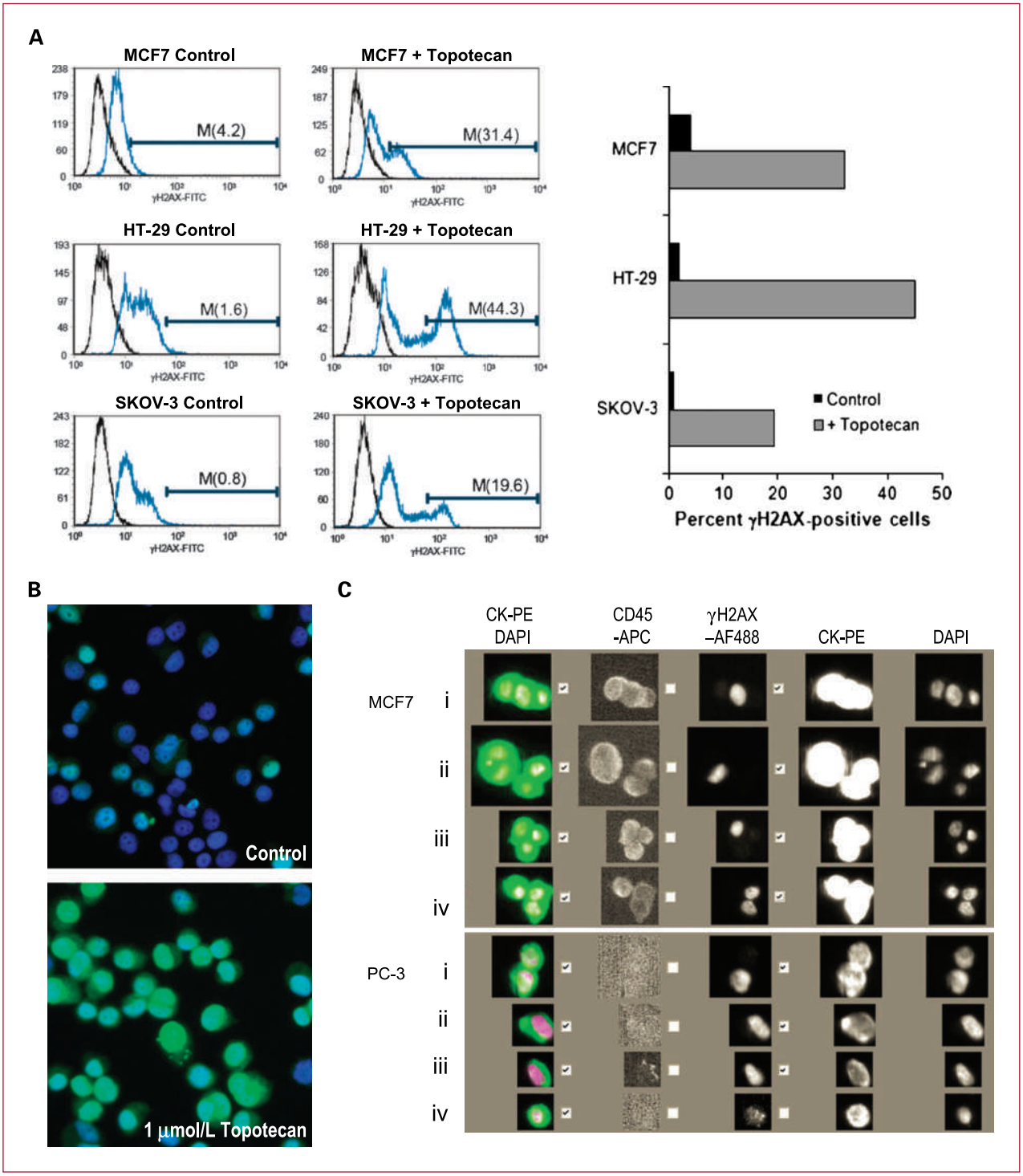

Fig. 1. Induction of γH2AX can be detected in topotecan-treated tumor cell lines and whole blood samples spiked with treated tumor cell lines using the γH2AX-AF488 antibody. A, FACS analysis of untreated (left) or 1 μmol/L topotecan-treated (right) MCF7, HT-29, and SKOV-3 tumor cells. FACS traces of γH2AX-AF488 (blue) and isotype control (black). Histogram presented for quantified FACS data from a representative experiment; data from three independent experiments were collected. B, photomicrographs of control and 1 μmol/L topotecan-treated MCF7 cells stained with γH2AX-AF488 (green) and DAPI (blue). Magnification, ×200. C, MCF7 or PC-3 cells were treated with 1 μmol/L topotecan for 2 h, spiked into whole blood from healthy donors, and stained using antibodies against CK-PE, CD45-APC, γH2AX-AF488, and DAPI. Checkboxes adjacent to images: a checked box next to the composite image (CK-PE/DAPI) indicates a CTC, a checked box next to the CD45-APC image indicates the presence of a contaminating leukocyte, and a checked box next to an image for γH2AX-AF488 staining indicates a γH2AX-positive cell. Four representative fields of cells isolated using the CellTracks AutoPrep and detected with the CellTracks Analyzer II are presented for each cell line (i-iv).

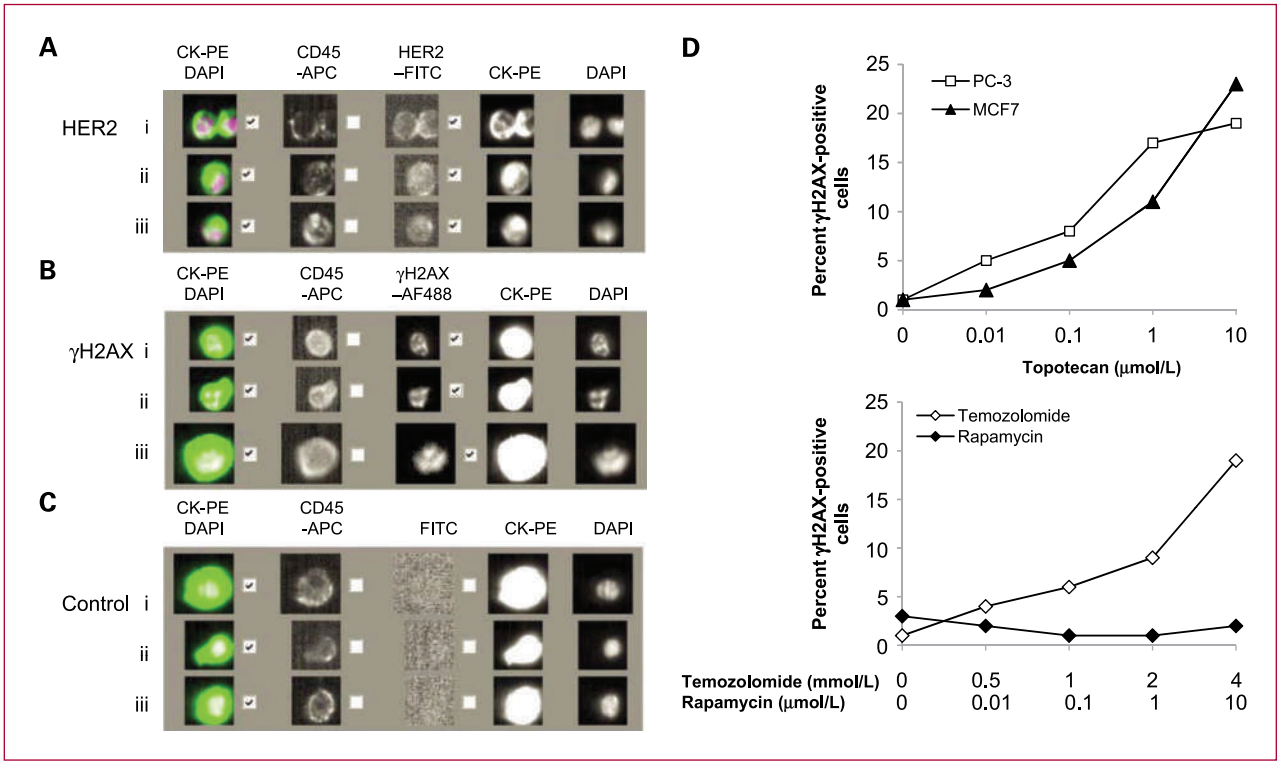

Fig. 2. γH2AX-AF488 staining was nuclear specific and DNA damage specific and increased in a dose-dependent manner in cells isolated from whole blood from healthy donors spiked with topotecan-treated tumor cell lines. A to C, representative images of three different cell fields captured using the CellTracks Analyzer II from cells stained with HER2-FITC, γH2AX-AF488, or no antibody (control) using the online staining protocol. SKOV-3 cells were treated with 1 μmol/L topotecan for 2 h and stained with anti-HER2-FITC (A), and 1 μmol/L topotecan-treated MCF7 cells were stained with γH2AX-AF488 (B). Control MCF7 cells were treated with 0.1 to 100 μmol/L topotecan for 2 h and processed without antibody. 100 μmol/L (C). Checkboxes adjacent to images: CTCs have a check next to the composite CK-PE/DAPI image and contaminating leukocytes have a check next to the CD45-APC images. Checks next to images of antibody-specific staining of HER2-FITC or γH2AX-AF488 indicate positive staining. D, MCF7 or PC-3 cells were treated with 0.01 to 10 μmol/L topotecan for 2 h, spiked into whole blood from healthy donors, and processed in the CellSearch system with anti-γH2AX-AF488 (top). One representative experiment. PC-3 cells were treated with 0.5 to 4 mmol/L temozolomide or 0.01 to 10 μmol/L rapamycin for 2 h, spiked into whole blood from healthy donors, and then processed in the CellSearch system. The isolated cells were stained with anti-γH2AX-AF488 (bottom).

particular experiment. The entire slide was visually inspected and counted to determine all nuclei and fraction of cells that are γH2AX positive, creating a baseline exposure specific to each experiment. The resulting monocaptures (DAPI and γH2AX) were merged into a single red-green-blue image for analysis.

Statistical analysis of percent γH2AX-positive CTCs per total CTCs recovered. The observed number of γH2AX-positive cells isolated from the total population of purified CTCs in each patient was measured. The two-sided 95% confidence intervals for the observed percentage positive were calculated using the exact binomial method (40). These confidence intervals may be calculated irrespective of the imprecision in the count data and irrespective of the varying CTC recovery rates for varying numbers of observed CTCs per aliquot, assuming that such imprecision and variability is independent of γH2AX positivity. The upper and lower 95% confidence interval bounds also correspond to 97.5% one-sided upper and lower confidence bounds, respectively.

Statistical analysis. Mean, SD, and coefficient of variation (CV; %) were determined using Microsoft Excel software.

Results

Detection of γH2AX in single CTCs

Flow cytometry and fluorescence microscopy were used to verify the feasibility of using γH2AX-AF488 for detection of γH2AX-positive cells following drug treatment. MCF7, HT-29, and SKOV-3 cells treated for 2 h with a therapeutically relevant concentration of topotecan (1 μmol/L) were analyzed for γH2AX-AF488 signal. Only 1% to 4% of untreated cells were γH2AX positive by fluorescence-activated cell sorting (FACS) analysis, whereas topotecan treatment increased the number of γH2AX-positive cells in all three tumor cell lines (MCF7, 31.4%; HT-29, 44.3%; and SKOV-3, 19.6%; Fig. 1A). In addition, fluorescence microscopy of topotecan-treated MCF7 cells revealed an increased number of γH2AX-positive cells compared with controls (Fig. 1B).

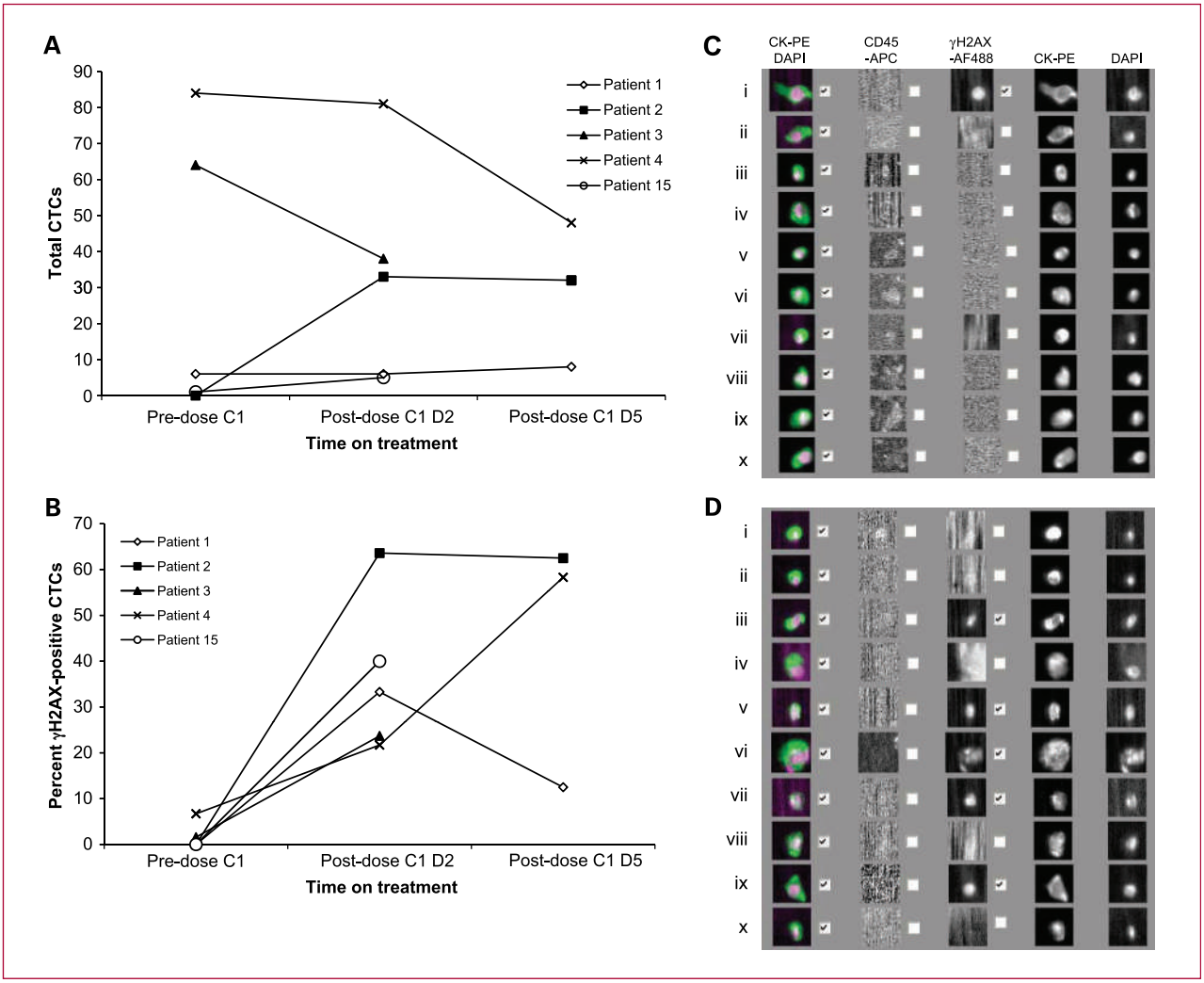

Fig. 3. Real-time detection of chemotherapy-induced time-course changes in γH2AX: correlation between total number of CTCs (A) and percent γH2AX-positive CTCs (B). Data presented for five patients on three different chemotherapeutic regimens (Table 3). For patient 4, 10 representative pretreatment (C) and 10 post-treatment (D) CTC images captured with the CellTracks Analyzer II are presented (i-x). Patient blood samples enriched for CTCs were stained using antibodies for CK-PE, CD45-APC, DAPI, and γH2AX-AF488. Checkboxes adjacent to images: a checked box next to the composite image (CK-PE/DAPI) indicates a CTC, a checked box next to the CD45-APC images indicates the presence of a contaminating leukocyte, and a checked box next to an image for γH2AX-AF488 staining indicates a γH2AX-positive cell.

Because neither flow cytometry nor fluorescence microscopy can be directly used to distinguish CTCs in whole blood samples, we explored whether the CellSearch system could be used in conjunction with γH2AX-AF488 antibody staining to identify γH2AX-positive CTCs from whole blood samples spiked with topotecan-treated cancer cell lines of epithelial origin. PC-3 and MCF7 cells were treated for 2 h with 1 μmol/L topotecan. Cells were then fixed, permeabilized, spiked into whole blood from healthy donors, and processed using the CTC detection and online γH2AX antibody staining protocols in the CellSearch system (unless otherwise noted, all spiked samples were processed in this manner). Individual γH2AX-positive cells were isolated and identified by the instrument from both MCF7-spiked and PC-3–spiked samples (Fig. 1C). These findings show that use of the CellSearch system combined with antibody staining using γH2AX-AF488 can directly detect topotecan-induced γH2AX signal in isolated CTCs.

Characterization of γH2AX signal in CTCs

Cellular localization of the γH2AX-AF488 signals from CellSearch-processed, topotecan-treated cells were compared with the plasma membrane marker HER2. MCF7 and SKOV-3 cells were treated with 1 μmol/L topotecan for 2 h and spiked into whole blood from healthy donors. SKOV-3–spiked samples were processed online with anti-HER2-FITC, and MCF7 samples were processed with anti-γH2AX-AF488 (Fig. 2A and B, respectively). As expected, HER2 signal was localized to the cell surface,

whereas γH2AX signal was solely in the nucleus and colocalized with DAPI staining. FITC channel background levels were also determined in topotecan-treated MCF7 cells. MCF7 cells were treated with 0.1, 1.0, 10, and 100 μmol/L topotecan for 2 h, spiked into whole blood, and processed with the CTC detection protocol in the absence of additional antibody. No signal was observed in the FITC channel from spiked MCF7 cells treated with any dose of topotecan, indicating neither topotecan nor the typical CTC staining process of CellSearch system affects the FITC channel signal background (Fig. 2C). These data indicate that processing of isolated CTCs with the CellSearch system and the γH2AX-AF488 antibody yields nuclear-specific γH2AX signal.

We next investigated whether topotecan induced γH2AX signal in a dose-dependent manner. MCF7 or PC-3 cells were treated with 0.01 to 10 μmol/L topotecan for 2 h, spiked into whole blood from healthy donors, and processed using the CellSearch system. In both cell lines, γH2AX signal increased in a dose-dependent manner (Fig. 2D). To verify that in our system the γH2AX signal observed from topotecan treatment was specific for DNA damage, we treated cells with two different chemotherapeutic agents. Temozolomide, a traditional DNA-damaging chemotherapeutic agent, and rapamycin, an antitumor proliferation drug that does not induce DNA damage, were assessed for their ability to induce H2AX phosphorylation in CTCs. PC-3 cells were treated with temozolomide (0.5, 1, 2, and 4 mmol/L) or rapamycin (0.01, 0.1, 1, and 10 μmol/L) for 2 h, spiked into whole blood from healthy donors, and stained for γH2AX-AF488. As expected, temozolomide, but not rapamycin, induced γH2AX accumulation in the isolated PC-3 tumor cells in a dose-dependent manner (Fig. 2D). Similar results were obtained from blood samples drawn from a patient with prostate cancer before and after 5 days of treatment with 2.2 mg/d topotecan. The representative images are shown in Fig. 3C and D (details described in Increased γH2AX-Positive CTCs following Treatment in Patient Samples). These findings confirm the specificity of the nuclear γH2AX signal detected in isolated CTCs.

Minimal assay variation in detection of CTCs and γH2AX-positive CTCs

We determined the accuracy and precision of detection of γH2AX signal in individual CTCs, including CTC recovery and reproducibility of analysis of γH2AX signal (Supplementary Material). Assay validation included CTC recovery studies using the CellSearch CTC Control kit and specific γH2AX assay variation studies with topotecan-treated tumor cell lines that were fixed, permeabilized, and spiked into whole blood samples from healthy donors.

Spike recovery experiments, incorporating the γH2AX immunofluorescence staining steps, yielded a recovery of 84% (average of six determinations; Supplementary Material). The lower recovery was not statistically significant but did show a consistently lower number of cells than the expected range, suggesting some cell loss in processing. However, the mean estimated recovery was

Table 1. Dose dependence and intraday and interday variation in γH2AX-positive CTCs purified from healthy donor blood samples spiked with topotecan-treated PC-3 cells

	γH2AX-positive CTCs (%)				
Topotecan (μmol/L)	0	0.01	0.1	1	10
Intraday variation*					
Sample 1	2	ND	30	37	45
Sample 2	1	ND	33	41	45
Sample 3	1	ND	34	39	43
Mean ± SD	1 ± 0.6	ND	32 ± 2.1	39 ± 2.0	44 ± 1.2
CV (%)	43.3	ND	6.4	5.1	2.6
Interday variation†					
Day 1	3	17	30	42	43
Day 2	4	18	31	42	45
Day 21	1	8	34	39	43
Mean ± SD	3 ± 1.5	14 ± 5.5	32 ± 2.1	41 ± 1.7	44 ± 1.2
CV (%)	57.3	38.4	6.6	4.2	2.6

Abbreviation: ND, not determined.

*Intraday variation of γH2AX signal from isolated CTCs represents the average of three independent samples processed by the same operator.

†Interday variation of γH2AX signal from isolated CTCs represents the average of three independent samples processed on three separate days by the same operator.

Table 2. Total CTC counts and γH2AX-positive CTCs before treatment in patients with advanced refractory disease

Patient	Cancer type	Stage	Total CTCs (7.5 mL blood)	Absolute γH2AX-positive CTCs	γH2AX-positive CTCs (%)
1	Breast	IV	6	0	0
2	Breast	IV	0	0	0
3	Small cell lung cancer	IV	64	1	1.6
4	Neuroendocrine prostate	IV	84	6	7.1
5	Colorectal	IV	10	1	10
6	Colorectal	IV	29	9	31
7	Colorectal	IV	4	0	0
8	Colorectal	IV	0	0	0
9	Colorectal	IV	4	0	0
10	Colorectal	IV	23	6	26.1
11	Hepatobiliary	IV	2	0	0
12	Appendiceal	IV	0	0	0
13	Esophageal	IV	0	0	0
14	Pancreatic	IV	4	1	25
15	Pancreatic	IV	1	0	0

the same as reported by Allard et al. for recoveries in the same range of spiked cell numbers (19).

Intraday, interday, and interoperator variation in CTC recovery. The CellSearch CTC Control kit containing two populations of a fixed breast cancer cell line at high and low densities was used for the recovery studies. High-density control samples ranged from 673 to 1,403 cells (mean, 1,038), and low-density control samples ranged from 16 to 74 cells (mean, 45). The control samples were first processed using the online CTC detection protocol with the CellSearch system to isolate control CTCs and obtain a total isolated CTC count. To simulate the offline process of γH2AX-AF488 staining that will be used to process patient samples, the isolated control CTCs were manually washed three times in the supplied cartridge inserted within the MagNest cassette. Cells were then resuspended, magnetically aligned within the MagNest cassette, and rescanned by the CellTracks Analyzer II to determine the post-wash recovery CTC count. Intraday and interday measurements were done by the same operator under the same conditions. For the low-density control, the CV was 6.8% for intraday and 22.7% for interday processed samples (Supplementary Table S1). High-density controls had a CV of 13.4% for intraday processed samples and 9.4% for samples processed on separate days. These results established intraday and interday assay consistency following offline processing and recovery of mock antibody-stained CTCs.

Using the CellSearch CTC Control kit, the reproducibility of CTC analysis performed by different operators was also examined. Duplicate control samples were processed independently by two different operators; interoperator variability was 10.7% for the low-density control and 3.0% for the high-density control, indicating minimal operator variability in sample processing (Supplementary Table S1; Operator 1 and Operator 2).

Intraday and interday variation in γH2AX-positive cell recovery. For evaluation of intraday assay variations on more clinically relevant samples and in the context of γH2AX induction, PC-3 cells were treated with 0.01, 0.1, 1.0, and 10 μmol/L topotecan for 2 h, spiked into whole blood, processed in the CellSearch system, and stained with anti-γH2AX-AF488 using the offline protocol. A dose-dependent γH2AX response to topotecan treatment was observed, and at all topotecan concentrations, an equivalent ratio of γH2AX-positive CTCs was recovered (CV <10%; Table 1). The interday variability for topotecan-treated cells (0.1-10 μmol/L) was also assessed by performing the same assay on consecutive days; again, minimal variability in recovered γH2AX-positive CTCs was observed (CV <10%; Table 1). Although γH2AX interday variability for cells treated with 0.01 μmol/L topotecan was high (CV, 38.4%), the variation is likely due to a difference in the effects of low-dose topotecan treatment on different passage numbers of cells.

Increased γH2AX-positive CTCs following treatment in patient samples

Using the validated assay method, whole blood samples were drawn from 15 patients with a variety of advanced malignancies enrolled in phase I clinical trials and baseline CTC values were determined. All patients had advanced refractory disease, and at first restaging (after two cycles of treatment), disease had progressed in all patients. Baseline CTC counts ranged from 0 to 84 cells per patient sample with 0% to 31% γH2AX-positive CTCs in untreated patients with stage IV cancers

such as breast, small cell lung, small cell prostate, and colorectal cancers (Table 2). Interday variation in CTC counts of patients at baseline was also examined to determine if intrapatient variability might influence total CTC counts. CTCs were collected from eight patients in two to four blood draws over a 1- to 60-day time span from the initial blood draw. Interday variation in CTC recovery within patients was <20% (Supplementary Fig. S1).

The drug-induced time-course changes in both total number of CTCs and the percent of CTCs expressing γH2AX were monitored to explore the correlation between total CTC count and γH2AX in patient CTCs during treatment. Figure 3 and Table 3 show the data from five patients on three different chemotherapeutic regimens: topotecan alone, a combination of an investigational inhibitor of poly(ADP-ribose) polymerase (PARP) and topotecan, or a combination of PARP inhibitor and cyclophosphamide. Increased numbers of γH2AX-positive cells were identified in all post-treatment samples during cycle 1 (Fig. 3B), whereas the change in the number of CTCs post-treatment varied from patient to patient (Fig. 3A; Table 3). All patients had progressive disease at the first restaging (after two cycles). One patient with stage IV neuroendocrine prostate cancer who underwent topotecan treatment (patient 4, Table 3) had some symptomatic improvement after the first cycle, but there was no evidence of tumor response by computed tomography scan. The patient received intravenous topotecan (1.2 mg/m^2/d) for 5 days in cycle 1. The percentage of γH2AX-positive CTCs increased during cycle 1 (7% pre-dose, 22% on day 2, and 58% on day 5), and by the fifth day, the number of isolated CTCs was decreasing (Fig. 3; Table 3). During cycle 2 (topotecan dose reduced to 1.0 mg/m^2/d for 5 days due to thrombocytopenia), the number of isolated CTCs increased during treatment (11 cells on day 1, 66 cells on day 2, and 119 cells on day 5); however, the percentage of γH2AX-positive cells remained high (40% on day 1, 33% on day 2, and 26% on day 5). These findings indicate that changes in γH2AX in CTCs, compared with simple CTC counts alone, may be used as a sensitive biomarker for monitoring the pharmacodynamic effects of DNA-damaging chemotherapeutic agents.

Table 3. γH2AX-positive CTC results from patients treated in three different chemotherapy clinical trials

Patient*	Cancer type	Time point	Total CTCs (7.5 mL blood)	Absolute γH2AX-positive CTCs	γH2AX-positive CTCs (%)	Lower-upper 95% confidence interval bound†
Topotecan						
4‡	Neuroendocrine prostate	Pre-dose C1	84	6	7.1	2.7-14.9
		Post-dose C1 D2	81	18	22.2	13.7-32.8
		Post-dose C1 D5	48	28	58.3	43.2-72.4
		Pre-dose C2	11	4	36.4	10.9-69.2
		Post-dose C2 D2	66	22	33.3	22.2-46
		Post-dose C2 D5	119	31	26.1	18.4-34.9
Topotecan + PARP inhibitor						
3	Small cell lung	Pre-dose C1	64	1	1.6	0-8.4
		Post-dose C1 D2	38	9	23.7	11.4-40.2
Cyclophosphamide + PARP inhibitor						
1	Breast	Pre-dose C1	6	0	0	0-45.9
		Post-dose C1 D2	6	2	33.3	4.3-77.2
		Post-dose C1 D5	8	1	12.5	0.3-52.7
2	Breast	Pre-dose C1	0§	0	0	Not valid
		Post-dose C1 D2	33	21	63.6	45.1-79.6
		Post-dose C1 D5	32	20	62.5	43.7-78.9
15	Pancreatic	Pre-dose C1	1§	0	0	0-97.5
		Post-dose C1 D2	5	2	40	5.2-85.3

Abbreviations: C, cycle; D, day.

*Patient number corresponds to the same patients referenced in Table 2. All patients had advanced refractory disease. No significant clinical responses were observed.

†Two-sided 95% confidence intervals for the observed percentage γH2AX-positive CTCs were calculated using the exact binomial method.

‡Patient 4 had some clinical (symptomatic) improvement after cycle 1, but there was no objective evidence of tumor response.

§The Poisson confidence limits range from 0 to 3.7 for a count of 0 total CTC and from 0 to 5.6 for a count of 1 total CTC.

Discussion

In this study, we present a new methodology for using γH2AX as a biomarker to measure drug effects on CTCs from patients during treatment with DNA-damaging agents. γH2AX is a well-established nuclear biomarker for DSB damage (22–24). Currently available CTC assay systems are limited to cell membrane antigens, such as HER2, MUC1, and epidermal growth factor receptor. In addition, currently used methods for detection of DNA damage in patient samples, including γH2AX immunofluorescence, alkaline comet assay, immunohistochemistry, and FACS analysis, can be labor- and time-intensive and have limited applicability in the clinic to monitor tumor response to chemotherapeutic agents. For instance, although FACS analysis of γH2AX is more sensitive than the alkaline comet assay (41, 42), it does not distinguish CTCs from normal blood cells, and the analytic sensitivity is too low to detect CTCs even if it could distinguish them; therefore, γH2AX response to drug treatment could be established only in nontumor cells (e.g., peripheral blood mononuclear cells). Compared with other approaches, the combination of CTC enrichment with nuclear γH2AX detection is not only a unique and advanced technique but also feasible for incorporation into clinical trials of molecularly targeted oncology drugs.

Validation studies, including CTC recovery, assay accuracy, and assay precision, show that this is a reproducible, reliable, and quantitative method (Supplementary Material). As with previous studies (19, 20), minimal variation in detection of CTCs from duplicate samples is observed. In addition, assay variation in detection of γH2AX-positive CTCs induced with therapeutically relevant concentrations of topotecan was <10%.

Studies employing cancer cell lines treated with increasing doses of topotecan and then spiked into whole blood showed that the fraction of γH2AX-positive cells increased in a dose-dependent manner, and treatment of cancer cell lines with rapamycin showed the specificity of the γH2AX response for DNA damage. In addition, our validation studies determined that there was an expected high variation and low γH2AX-positive cell recovery when initial CTC recovery was low but minimal day-to-day variation in CTC recovery (<20%) when CTC counts were >10 cells per sample. For those patients with low to no CTCs, it may be necessary to find alternate capture antibodies for evaluation.

Using this validated assay, we performed a population baselining study to assess the range of CTC counts in our patients and the day-to-day variability in CTC counts in single patients. Initial CTC counts were determined for 15 patients with a variety of cancer types; 73% (11 of 15) of the patients had measureable CTCs in their blood before treatment. We also determined that following CTC enrichment with the CellSearch system, staining with the γH2AX-AF488 antibody yielded a clear nuclear signal and that the fraction of γH2AX-positive CTCs was low and consistent in patients not undergoing radiation treatment or chemotherapy.

We then evaluated patients on various chemotherapy regimens and found that the fraction of γH2AX-positive CTCs increased in patients treated with topotecan alone, a combination of topotecan and a PARP inhibitor, or a combination of cyclophosphamide and PARP inhibitor. Increased numbers of γH2AX-positive cells can be measured irrespective of increases or decreases in the total number of recovered CTCs. The change in the number of CTCs induced by drug treatment varied from patient to patient and may be dependent on individual short-term drug response. In the case of patients with high baseline CTC counts, CTC counts decreased following treatment. However, for patients with zero or low baseline CTCs, CTC counts increased post-treatment. In this second group of patients, it appears that CTCs were shed from the tumor in response to therapy. If this interpretation is correct, the γH2AX response detected in CTCs was not likely due to CTCs already in the peripheral blood being exposed to the drug but instead directly reflected the drug-induced DNA damage in cells in the tumor.

Meng et al. estimated CTC half-life by flow cytometry using specimens from five breast cancer patients presurgery and perisurgery (43). All five patients had declining numbers of CTCs postsurgery, with 6 of 10 aggregated data points falling into the background noise level of the instrument within the first 24 h. Their conclusion of a CTC half-life measured in hours is neither supported nor contradicted by our data, because the majority of patients in our study had increased CTC numbers post-chemotherapy. However, if their conclusion is correct, the half-life estimate would strengthen our argument that γH2AX-positive CTCs collected 24 h after a single administration of drug are recently shed from the tumor and reflect a pharmacodynamic response of the tumor. It should be noted that the patients in our study all have active disease (with distant metastases) that has failed numerous rounds of therapy. Thus, our current data are limited to patients in phase I trials, which may not be a fair representation of the overall patient population in oncology. It is possible that patients with disease that has not responded to multiple lines of therapy have CTCs with different cell surface markers. We are exploring whether markers other than EpCam will isolate more CTCs from patient blood samples at the National Cancer Institute. It remains to be established, however, whether the fraction of γH2AX-positive CTCs is representative of the positive fraction in the tumor, and this will be investigated in upcoming clinical trials. In addition, the half-life of CTCs in peripheral blood is not known and needs to be determined once a suitable method has been developed.

Based on our current data, several additional questions and limitations need to be considered. First, the current assay is limited to chemotherapeutic agents that induce a DNA damage response through the ataxia telangiectasia mutated/γH2AX pathway; however, alternate nuclear biomarkers can be used to expand this approach (11, 12, 16). Second, while the number of isolated CTCs is currently

used as a prognostic indicator for disease outcome in patients with breast and colon cancer, the utility of monitoring changes in the number of γH2AX-positive CTCs during treatment and the types of cancers this technology can be reliably employed to monitor are yet to be determined and will require broader testing. Finally, it is unknown whether γH2AX in CTCs will correlate with clinical efficacy (tumor shrinkage). To address these last points, this assay is currently being incorporated as a correlative study in early-phase clinical trials at the National Cancer Institute, and drug effect on disease progression will be correlated to γH2AX-positive CTCs.

In conclusion, γH2AX is a sensitive biomarker for monitoring the pharmacodynamic effects of topotecan alone, a combination of topotecan and PARP inhibitor, or a combination of PARP inhibitor and cyclophosphamide in isolated CTCs. This method only requires patient blood samples, allowing the possibility of monitoring drug effect throughout treatment. This method has obvious advantages over invasive tumor biopsies, as well as tumor imaging, which often requires a significant span of time between images to detect effects from treatment. In combination with CTC counts, monitoring of γH2AX signal can lead to enhanced monitoring of chemotherapeutic effects in the clinic. In ongoing clinical trials within the National Cancer Institute, we are testing whether assaying γH2AX levels in CTCs provides a more sensitive marker for assessing pharmacodynamic effects in patients than total CTC counts alone. Another potential application of this method is to determine the half-life of CTCs after shedding from the tumor; such measurements could have both theoretical and diagnostic significance. Finally, the strategy employed for our method can be adapted for additional nuclear biomarkers in CTCs. We hope this will become a useful tool to evaluate pharmacodynamic effects in clinical trials of novel DNA-damaging agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Dowlati A, Haaga J, Remick SC, et al. Sequential tumor biopsies in early phase clinical trials of anticancer agents for pharmacodynamic evaluation. Clin Cancer Res 2001;7:2971–6.
2. Helft PR, Daugherty CK. Are we taking without giving in return? The ethics of research-related biopsies and the benefits of clinical trial participation. J Clin Oncol 2006;24:4793–5.
3. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 2005;23:1420–30.
4. Hayes DF, Smerage J. Is there a role for circulating tumor cells in the management of breast cancer? Clin Cancer Res 2008;14:3646–50.
5. Smerage JB, Hayes DF. The prognostic implications of circulating tumor cells in patients with breast cancer. Cancer Invest 2008;26:109–14.
6. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781–91.
7. Hayes DF, Cristofanilli M, Budd GT, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 2006;12:4218–24.
8. Cohen SJ, Punt CJ, Iannotti N, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:3213–21.
9. Meng S, Tripathy D, Shete S, et al. HER-2 gene amplification can be acquired as breast cancer progresses. Proc Natl Acad Sci U S A 2004;101:9393–8.
10. Bolke E, Orth K, Gerber PA, et al. Gene expression of circulating tumour cells and its correlation with tumour stage in breast cancer patients. Eur J Med Res 2009;14:359–63.
11. Smirnov DA, Foulk BW, Doyle GV, Connelly MC, Terstappen LWMM, O'Hara SM. Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. Cancer Res 2006;66:2918–22.
12. Smirnov DA, Zweitzig DR, Foulk BW, et al. Global gene expression profiling of circulating tumor cells. Cancer Res 2005;65:4993–7.
13. Goodman OB, Jr., Fink LM, Symanowski JT, et al. Circulating tumor cells in patients with castration-resistant prostate cancer baseline values and correlation with prognostic factors. Cancer Epidemiol Biomarkers Prev 2009;18:1904–13.
14. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 2007;25:5287–312.
15. Alix-Panabieres C, Riethdorf S, Pantel K. Circulating tumor cells and bone marrow micrometastasis. Clin Cancer Res 2008;14:5013–21.
16. Sieuwerts AM, Kraan J, Bolt J, et al. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. J Natl Cancer Inst 2009;101:61–6.
17. Lang JE, Mosalpuria K, Cristofanilli M, et al. HER2 status predicts the presence of circulating tumor cells in patients with operable breast cancer. Breast Cancer Res Treat 2008;113:501–7.
18. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 2008;359:366–77.
19. Allard WJ, Matera J, Miller MC, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 2004;10:6897–904.
20. Riethdorf S, Fritsche H, Muller V, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a

validation study of the CellSearch system. Clin Cancer Res 2007;13:920–8.
21. Shaffer DR, Leversha MA, Danila DC, et al. Circulating tumor cell analysis in patients with progressive castration-resistant prostate cancer. Clin Cancer Res 2007;13:2023–9.
22. Huang X, Okafuji M, Traganos F, Luther E, Holden E, Darzynkiewicz Z. Assessment of histone H2AX phosphorylation induced by DNA topoisomerase I and II inhibitors topotecan and mitoxantrone and by the DNA cross-linking agent cisplatin. Cytometry A 2004;58:99–110.
23. Takagi K, Dexheimer TS, Redon C, et al. Novel E-ring camptothecin keto analogues (S38809 and S39625) are stable, potent, and selective topoisomerase I inhibitors without being substrates of drug efflux transporters. Mol Cancer Ther 2007;6:3229–38.
24. Zhao H, Traganos F, Darzynkiewicz Z. Kinetics of histone H2AX phosphorylation and Chk2 activation in A549 cells treated with topotecan and mitoxantrone in relation to the cell cycle phase. Cytometry A 2008;73A:480–9.
25. Lavin MF, Birrell G, Chen P, Kozlov S, Scott S, Gueven N. ATM signaling and genomic stability in response to DNA damage. Mutat Res 2005;569:123–32.
26. Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K, Bonner W. Histone H2A variants H2AX and H2AZ. Curr Opin Genet Dev 2002;12:162–9.
27. Chowdhury D, Keogh MC, Ishii H, Peterson CL, Buratowski S, Lieberman J. γ-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. Mol Cell 2005;20:801–9.
28. Kinders R, Hollingshead M, Lawrence S, et al. Development and validation of a quantitative assay for γH2AX in needle biopsies using topoisomerase I inhibitors. AACR Meet Abstr 2007;2007:C50.
29. Colvin OM. An overview of cyclophosphamide development and clinical applications. Curr Pharm Des 1999;5:555–60.
30. Hirose Y, Berger MS, Pieper RO. Abrogation of the Chk1-mediated G (2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. Cancer Res 2001;61:5843–9.
31. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 2000;10:886–95.
32. Clingen PH, Wu JYH, Miller J, et al. Histone H2AX phosphorylation as a molecular pharmacological marker for DNA interstrand crosslink cancer chemotherapy. Biochem Pharmacol 2008;76:19–27.
33. Pommier Y. Topoisomerase I inhibitors: camptothecins and beyond. Nat Rev Cancer 2006;6:789–802.
34. Strumberg D, Pilon AA, Smith M, Hickey R, Malkas L, Pommier Y. Conversion of topoisomerase I cleavage complexes on the leading strand of ribosomal DNA into 5′-phosphorylated DNA double-strand breaks by replication runoff. Mol Cell Biol 2000;20:3977–87.
35. Cai Z, Chen Z, Bailey KE, Scollard DA, Reilly RM, Vallis KA. Relationship between induction of phosphorylated H2AX and survival in breast cancer cells exposed to ^{111}In-DTPA-hEGF. J Nucl Med 2008;49:1353–61.
36. Kuo LJ, Yang LX. γ-H2AX—a novel biomarker for DNA double-strand breaks. In Vivo 2008;22:305–9.
37. Guirouilh-Barbat J, Redon C, Pommier Y. Transcription-coupled DNA double-strand breaks are mediated via the nucleotide excision repair and the Mre11-50-Nbs1 complex. Mol Biol Cell 2008;19:3969–81.
38. Tanaka T, Huang X, Dorota Halicka T, et al. Cytometry of ATM activation and histone H2AX phosphorylation to estimate extent of DNA damage induced by exogenous agents. Cytometry A 2007;71A:648–61.
39. Hollingshead M, Borgel S, Carter J, et al. Evidence of *in vivo* efficacy for the indenoisoquinolines linked with pharmacodynamic markers for γH2AX. AACR Meet Abstr 2007;2007:C18.
40. Snedecor GW, Cochran WG. Statistical methods. Ames (IA): Iowa State University Press; 1980.
41. Ismail IH, Wadhra TI, Hammarsten O. An optimized method for detecting γ-H2AX in blood cells reveals a significant interindividual variation in the γ-H2AX response among humans. Nucleic Acids Res 2007;35:e36.
42. Olive PL, Durand RE, Jackson SM, et al. The comet assay in clinical practice. Acta Oncol 1999;38:839–44.
43. Meng S, Tripathy D, Frenkel EP, et al. Circulating tumor cells in patients with breast cancer dormancy. Clin Cancer Res 2004;10:8152–62.