Rapid Flow Cytometry–Based Structural Maintenance of Chromosomes 1 (SMC1) Phosphorylation Assay for Identification of Ataxia-Telangiectasia Homozygotes and Heterozygotes

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BACKGROUND: No rapid reliable method exists for identifying ataxia-telangiectasia (A-T) homozygotes or heterozygotes. Heterozygotes are at an increased risk of cancer and are more sensitive to the effects of ionizing radiation (IR) than the general population. We report a rapid flow cytometry (FC)-based ataxia-telangiectasia mutated (ATM) kinase assay that measures ATM-dependent phosphorylation of structural maintenance of chromosomes 1 (SMC1) following DNA damage (FC-pSMC1 assay).

METHODS: After optimizing conditions with lymphoblastoid cell lines (LCLs), we studied peripheral blood mononuclear cells (PBMCs) isolated from 16 healthy donors (unknowns), 10 obligate A-T heterozygotes, and 6 unrelated A-T patients. One hour after DNA damage (by either IR or bleomycin), the cells were fixed and incubated with a primary antibody to SMC1pSer966. We analyzed the stained cells by FC to determine the difference in geometric mean fluorescence intensity (ΔGMFI) of untreated and treated cells; this difference was expressed as a percentage of daily experimental controls.

RESULTS: The FC-pSMC1 assay reliably distinguished ATM heterozygotes and homozygotes from controls. Average ΔGMFI percentages (SD) of daily controls were, for unknowns, 106.1 (37.6); for A-T heterozygotes, 37.0 (18.7); and for A-T homozygotes; 8.73 (16.2). Values for heterozygotes and homozygotes were significantly different from those of controls (P < 0.0001).

CONCLUSIONS: The FC-pSMC1 assay shortens the turnaround time for diagnosing A-T homozygotes from approximately 3 months to approximately 3 h. It also identifies A-T heterozygotes and can be used for prenatal counseling or for screening individuals in large study cohorts for potential ATM heterozygosity, which can then be confirmed by sequencing.

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Ataxia-telangiectasia (A-T)4 is a progressive neurodegenerative disorder of childhood onset (1, 2), inherited in an autosomal recessive pattern. The gene product defective in this syndrome is ATM (ataxia-telangiectasia mutated) kinase. After DNA damage, ATM phosphor-ylates >700 target proteins involved in cell-cycle checkpoints, apoptosis, nonsense-mediated decay, oxidative stress response, and DNA repair (3). These processes involve proteins such as protein 53 (p53), checkpoint kinase 2 (CHK2), Nijmegen breakage syndrome 1 (NBS1), structural maintenance of chromosomes 1 (SMC1), γ histone 2A variant X (γH2AX), Fanconi anemia complementation group D2 (FANCD2), and breast cancer susceptibility 1 (BRCA1) (4). Several groups of interacting proteins influence the crucial S phase checkpoint, such as the ATM/CHK2/Cdc25A, ATM/NBS1/SMC1, FANCD2-BRCA1, and RAD50/ MRE11/NBS1 complexes (5, 6). The phosphorylation of SMC1 by ATM kinase, after ATM kinase recruitment and activation by NBS1 and BRCA1 to DNA double strand break (DSB) damage sites, is thought to play an important role in the rapid cellular response to radiation damage.

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4 Nonstandard abbreviations: A-T, ataxia-telangiectasia; ATM, ataxia-telangiectasia mutated; p53, protein 53; CHK2, checkpoint kinase 2; NBS1, Nijmegen breakage syndrome 1; SMC1, structural maintenance of chromosomes 1; γH2AX, γ histone 2A variant X; FANCD2, Fanconi anemia complementation group D2; BRCA1, breast cancer susceptibility 1; DSB, double strand break; IR, ionizing radiation; LCL, lymphoblastoid cell line; CSA, colony survival assay; PBMC, peripheral blood mononuclear cell; FC-pSMC1, flow cytometry–based ATM-dependent phosphorylation of SMC1; GMFI, geometric mean fluorescence intensity; DC, daily control; WT, wild type.
Presently, diagnostic testing for A-T takes approximately 12 weeks and often is required on infants who are unable to provide more than a few milliliters of blood (7–9). The diagnostic protocol includes establishing a lymphoblastoid cell line (LCL) from whole blood, performing a colony survival assay (CSA) for radiosensitivity, and immunoblotting for ATM protein. We previously reported an ATM-ELISA assay that can be used to confirm a diagnosis of A-T within 2 days on small numbers of peripheral blood mononuclear cells (PBMCs) (10). Although the accuracy of the ATM-ELISA assay is >99%, it requires a renewable source of purified ATM protein as a standard (11). Because of the large size of the gene, the cost of sequencing approximately 15000 nt, the frequency of missed mutations (approximately 10%), and the limitations of sequence interpretation, direct ATM (ataxia-telangiectasia mutated gene) sequencing is not the recommended test of first choice for establishing a diagnosis; it is best reserved for confirmed A-T cases, in whom the consequences of specific mutations may influence both the rate of disease progression and future therapy (12, 13).

A-T heterozygotes are at an increased risk for breast cancer and possibly heart disease (14–25). Identifying heterozygosity in the absence of a prior affected family member is even more challenging. The goal in such cases is to establish whether a single ATM DNA change of consequence (i.e., a mutation) is present. ATM protein levels are usually 40%–50% of normal in heterozygotes but cannot be reliably quantified by immunoblotting or ATM-ELISA from a single peripheral blood sample (9, 10). Radiosensitivity (CSA) testing of cell lines from known A-T heterozygotes is usually inconclusive, yielding scores in the normal or intermediate range. The most rigorous efforts at heterozygote identification have never exceeded 80%–90% accuracy (14, 15, 25) and are not practicable for clinical testing.

The above factors prompted us to seek a rapid assay based on ATM function. The flow cytometry (FC)-based ATM kinase assay, which measures ATM-dependent phosphorylation of the protein SMC1 after DNA damage, greatly reduces the turnaround time necessary for identifying individuals with functionally compromised ATM protein kinase activity. The FC-PSMC1 assay could be performed in most clinical laboratories using 1–2 mL blood. We believe this to be a reliable, rapid screening assay for ATM homozygosity or heterozygosity.

Materials and Methods

BLOOD PROCESSING AND TRANSFORMING LYMPHOBLASTOID CELL LINES

After informed consent, we collected blood from 7 normal daily controls, 16 healthy volunteers (unknowns), 10 obligate heterozygotes, and 6 unrelated A-T patients. PBMCs were isolated by centrifugation over a Ficoll-Hypaque density gradient (Amersham Pharmacia Biosciences). Mononuclear cells were transformed with Epstein–Barr virus and maintained at 37 °C and 5% CO₂ in RPMI 1640 (Gibco Invitrogen) containing 15% heat-inactivated fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Gibco Invitrogen). ATM mutations for the A-T patients studied are listed in Supplemental Table 1, which accompanies the online version of this article at www.clinchem.org/content/vol55/issue3.

IMMUNOBLOT ANALYSIS

Nuclear extracts from 5–10 million LCLs were prepared following the manufacturer’s protocol (NE-PER Nuclear and Cytoplasmic Extraction Reagents). Nuclear lysate (25 μg) was electrophoresed on a 7.5% SDS polyacrylamide gel, transferred onto polyvinylidene difluoride membrane (Bio-Rad), blocked with 5% milk, and incubated with a 1:1000 dilution of rabbit anti-SMC1pSer966, rabbit anti-Mre11, rabbit anti-NBS antibody, or rabbit anti-ATM (Novus) overnight at 4 °C. Horseradish peroxidase–conjugated Ig anti-rabbit antibody was added at a dilution of 1:3000 and incubated at room temperature for 40 min. All proteins were detected using an enhanced chemiluminescence kit (Amersham Pharmacia).

FC-PSMC1

PBMCs or LCLs were suspended in PBS and split into two aliquots. To produce DNA damage, we irradiated the cells (10 Gy) or treated them with 1.5 μg/mL bleomycin. We incubated the cells at 37 °C in 5% CO₂ for 1 h, at which time they were fixed and permeabilized using Fix & Perm cell permeabilization kit (Caltag Laboratories; Invitrogen). Briefly, we used 100 μL fixation reagent A to resuspend, vortex-mix, and hold the cells for 3 min at room temperature, followed by the addition of 3 mL cold methanol. The methanol was added during vortex-mixing. The cells were incubated at 4 °C for 10 min and centrifuged at 300g for 5 min. After centrifugation, we removed the supernatants and washed the cells with 3 mL PBS + 0.1% sodium azide and 5% fetal bovine serum, followed by centrifugation for 5 min at 300g. We resuspended the cells in 100 μL permeabilization reagent B, added 5 μg rabbit anti-SMC1pSer966 antibody (NB100–206; Novus), and incubated the preparation for 50 min at room temperature. After incubation, we added 3 mL wash buffer and centrifuged the cells for 5 min at 300g. We removed the supernatant, resuspended the cells in 100 μL PBS containing 5 μg antirabbit-lg fluorescein isothiocyanate–conjugated antibody (Jackson Immunoresearch Laboratories), and incubated them in the dark for 45 min at
20 °C. The cells were washed with 3 mL wash buffer, centrifuged for 5 min at 300g, resuspended in PBS, and fixed with 0.1% paraformaldehyde.

FC-pSMC1 DATA ANALYSIS
We analyzed samples using a FACScaliber (BD Biosciences) with Cell Quest software, which plots geometric mean fluorescence intensity (GMFI) on the x axis using a log scale. GMFI peaks were converted to a linear scale for calculating % of daily control (%DC). The mean GMFI peak (linear scale) of untreated cells was subtracted from calculating % of daily control (%DC). The mean GMFI of a healthy daily control (DC) and the ΔGMFI for all samples were normalized against the ΔGMFI of a healthy daily control (DC) and expressed as a proportion (%DC).

PRECISION STUDIES
We determined the intraday assay (within-run) variability, expressed as a CV for the FC-pSMC1 assay, by testing PBMCs isolated from 1 healthy donor and 1 A-T heterozygote donor, a total of 10 times on the same day. For interday assay (between-day) variability (CV), we collected PBMCs from the same healthy donor used in the intraday studies on 5 different days, and each sample was assayed singly each day.

ATM-ELISA
ATM-ELISA has been described previously (10). Briefly, we prepared nuclear lysates from LCLs and PBMCs by use of NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the manufacturer’s instructions. We measured the protein concentrations of the nuclear lysates using a modified Bradford method (Bio-Rad Laboratories), and we used 100 μg nuclear lysate for ATM protein quantification by immunoassay. Flat-bottomed, 96-well, high-binding enzyme immunoassay/RIA plates (Corning) were incubated with a purified mouse monoclonal antibody, ATM-2C1 (GeneTex), at 10 mg/L in PBS (pH 7.4) in a final volume of 120 μL in PBS (pH 7.4) for 6 h at room temperature. After washing, the plate was blocked with PBS containing 30 g/L BSA and 1 mL/L Tween 20 for 45 min. We added purified ATM protein (serial 2-fold dilutions starting at 640 μg/L) in triplicate and unknown nuclear-cell/whole-cell lysates in duplicate. Calibrators and unknown samples were added in a total volume of 120 μL, with PBS containing 10 g/L BSA and 1 mL/L Tween 20 used as diluent. The plate was incubated overnight at room temperature, washed, and blocked; rabbit anti-ATM affinity-purified antibody (400-fold dilution in 120 μL volume; Novus Biologicals) was then added and incubated for 3 h at room temperature. After washing, the plate was incubated with horseradish peroxidase–conjugated goat antirabbit IgG antibody (4000-fold dilution; Jackson ImmunoResearch Laboratories.) for 3 h at room temperature. After the plate was washed, 100 μL tetramethylbenzidine substrate (1-step Turbo TMB-ELISA; Pierce Biotechnology) was added to each well and incubated for 25 min; sulfuric acid (1 mol/L) was added to stop color formation and produce a yellow color. We measured the absorbance of each well at a wavelength of 450 nm and subtracted background absorbance at 630 nm. We generated a calibration curve using a linear curve-fitting program with a log-log scale (Microplate Manager Program; Bio-Rad), and ATM concentrations of unknown samples were determined from the calibration curve.

Results

DETECTION OF ATM KINASE ACTIVITY BY SMC1 PHOSPHORYLATION, USING LCLs
SMC-1 protein is directly phosphorylated by ATM protein on serine residues 957 and 966 in response to DNA damage (4–6). Fig. 1A demonstrates by immunoblotting that the phosphorylation of SMC1 on serine 966 after ionizing radiation (IR) (10 Gy) is absent in ATM-deficient cells (AT153LA) and reduced in an A-T heterozygote cell (ATHET4), compared with wild-type (WT) cells (NAT9). In addition, ATM protein levels are absent in A-T cells and reduced in heterozygotes (Fig. 1A). We hypothesized that SMC1 phosphorylation post-IR should also be observable by flow cytometry if nuclear proteins could be adequately stained after permeabilization of cell membranes. As shown in Fig. 1B, a shift in GMFI was observed in NAT9 (wild type) when pre- and post-IR cells were compared. No shift was observed after IR in AT153LA, and a reduced shift (compared with WT) was observed in ATHET4.

We extended these experiments to LCLs from 7 healthy unknowns, 4 A-T heterozygotes, and 10 A-T LCLs. We observed an average IR-induced response of unknowns, 89.9%DC (9.2%DC); A-T heterozygotes, 58.1%DC (14.4%DC); A-T homozygotes, 0.83%DC (3.3%DC) (i.e., no shift) (Fig. 1C). The ΔGMFIs between genotypes were significantly different from each other (P = 5 × 10−8, Kruskal–Wallis test). No shift was observed using an irrelevant antibody to aprataxin, another nuclear protein (data not shown).

DETECTION OF ATM KINASE ACTIVITY BY SMC1 PHOSPHORYLATION, USING PBMCs
We next tested the assay on fresh peripheral blood mononuclear cells (PBMCs). When initial results were noted to be comparable to those using LCLs, all subsequent experiments were performed using PBMCs. Various IR doses were examined; we irradiated PBMCs with 5, 10, or 20 Gy and established that a 10 Gy IR dose optimized increases in the ΔGMFI (data not shown). Dilutions of both the primary and secondary antibodies were also further optimized.

Clinical Chemistry 55:3 (2009) 465
We evaluated whether the FC-pSMC1 assay would be sufficiently sensitive for distinguishing obligate A-T heterozygotes (i.e., parents of A-T patients) from WT and A-T homozygotes. As shown in Fig. 2A, the shift for both parents was less than that of WT cells but clearly more than the negligible shift seen with cells from the affected child (AT223LA). This was concordant with the previous immunoblot studies showing reduced amounts of ATM kinase activity in A-T heterozygotes (9) (Fig. 1A).

Fig. 1. Detection of IR-induced ATM-dependent phosphorylation of SMC1pSer966 using LCLs.

(A), Immunoblot of SMC1pSer966 for nuclear lysates before (−) and after (+) 10 Gy IR. SMC1 was used as a loading control. (B), FC-pSMC1 assay using WT (NAT9), A-T (AT153LA), and A-T heterozygote (ATHET4) LCLs. Filled peaks represent no IR and unfilled peaks represent post-IR cells. (C), FC-pSMC1 was performed on LCLs from 7 healthy unknowns (left), 4 A-T heterozygotes (ATHET 1–4) (center), and 10 A-T homozygotes (right). All experiments included a healthy daily control (DC) and all ΔGMFIs are expressed as a percentage of the DC. P values between each group are indicated above each panel. Solid lines represent average ΔGMFI as a percentage of the daily controls, and dashed lines represent SDs.

We next tested PMBCs from 16 unknowns, 10 obligate A-T heterozygotes, and 6 unrelated homozygotes. As can be seen in Fig. 2B, after 10 Gy IR, we observed an average response of 106.1% DC (37.6% DC). By comparison, when we tested fresh PBMCs isolated from the 10 obligate ATM heterozygotes, the average response to IR damage was significantly lower than that of healthy unknowns: 37.0% DC (18.7% DC) vs 106.1% DC (37.6% DC) (P < 0.006). Responses of both the unknowns and A-T heterozy-
gotes were significantly larger than those of A-T homozygotes: 106.1%DC (37.6%DC) vs 8.7%DC (16.2%DC), \((P < 0.001)\), and 37.0%DC (18.7%DC) vs 8.7%DC (16.2%DC), \((P < 0.001)\). None of the responses for A-T PBMCs fell within the ranges for unknowns or obligate A-T heterozygotes (Fig. 2B). The responses between genotypes were significantly different from each other \((P < 0.001\), Kruskal–Wallis test\). The FC-pSMC1 assay data for A-T cells did not appear to be influenced by \(ATM\) mutations (online Supplemental Table 1). We concluded that FC-pSMC1 was able to reliably distinguish between unknowns, A-T heterozygotes, and A-T homozygotes.

**BLEOMYCIN AS A SUBSTITUTE FOR IRRADIATION**

Because some clinical laboratories may not have access to a cell irradiator, we substituted bleomycin (a chemical inducer of double strand DNA breaks) for irradiation in the FC-pSMC1 assay (26). After optimizing bleomycin dosage conditions, we treated PBMCs from a normal control, a second healthy unknown (i.e., 2 healthy individuals, 1 predesignated to be the daily control; the other considered as an unknown), an A-T heterozygote, and an A-T homozygote for 1 h at 37°C with 1.5 \(\mu\)g/mL bleomycin instead of IR. The bleomycin treatment caused a shift in GMFI (i.e., the \(\Delta\)GMFI) that was comparable to those seen in PBMCs treated with IR: unknown, 96.7%DC; A-T heterozygote, 48.6%DC; and A-T homozygote, \(-9.2\)\%DC (i.e., no shift) (Fig. 2B). Because testing for most rare diseases is performed at a distant referral laboratory, blood samples are typically 1 to 3 days old when they are tested. In limited testing, we detected no discernible pattern of change in \%DC values of 2- or 3-day-old shipped samples using either IR or bleomycin (data not shown).

**PRECISION STUDIES**

To determine intraday assay (within-run) variance (CV), PBMCs from a healthy daily control, a healthy...
unknown, and an A-T heterozygote were isolated and assayed 10 times in 1 day by FC-pSMC1. The intraday assay variance (CV) was unknown, 27.5%DC, and A-T heterozygote, 17.4%DC (online Supplemental Table 2). The average increase in GMFIs as a percentage of the daily control for intraday sample variation was unknown, 96.2% DC (26.5%DC); A-T heterozygote; 40.8%DC (7.1%DC), (P < 0.001) (Fig. 3, online Supplemental Table 2). Interday assay (between-run) imprecision (CV) of samples drawn on 5 consecutive days was unknown, 9.4% DC (online Supplemental Table 2). The average increase in ΔGMFIs was 83.7%DC (7.9%DC) (Fig. 3, online Supplemental Table 2). In addition to the above studies, we also found that the assay could be performed with only 2 mL whole blood, allowing this assay to be used on very young children (data not shown).

**COMPARISON OF ATM ELISA AND FC-PSMC1**

We previously reported an ATM-ELISA assay that can be used to confirm a diagnosis of A-T on small numbers of PBMCs (10). Although the accuracy to identify ATM-deficient patients using the ATM-ELISA is >99%, limitations include that (1) a dependable source of purified ATM protein is required as a standard and (2) this test does not identify kinase-dead ATM protein, as encountered in some A-T patients. Furthermore, the variability of unbound ATM nuclear protein in fresh blood cells does not allow a reliable diagnosis of heterozygosity, as previously reported (10). Nonetheless, we wanted to determine whether the results from the FC-pSMC1 assay would be comparable to those seen when using ATM-ELISA. We performed ATM-ELISA using 100 μg nuclear lysates isolated from LCLs of 1 healthy unknown, 4 obligate A-T heterozygotes (ATHET 1–4), and 2 A-T patients (AT133LA, GRAT1), as well as a daily control LCL (Fig. 4B). On average, ATM protein levels (also calculated as a percentage of the daily control LCL) were the following: unknown, 92.0%; A-T heterozygotes, 50.9%; and A-T homozygotes 1.1% (Fig. 4B). These results were comparable to those seen using FC-pSMC1 (Fig. 4A): unknown, 97.3%DC (3.81%DC); A-T heterozygotes, 58.0%DC (14.4%DC); A-T homozygotes, 0.32%DC (0.35%DC). On the basis of these very limited studies, the FC-pSMC1 and ATM-ELISA assays could be used adjunctively to identify the A-T homozygosity and heterozygosity of LCLs.

**POTENTIAL FALSE-POSITIVES FOR OTHER GENOMIC INSTABILITY DISORDERS**

We evaluated whether the FC-pSMC1 assay might give false-positive results for related disorders, such as other radiosensitive disorders (Nijmegen breakage syndrome, Mre11 deficiency, DNA ligase IV deficiency, Fanconi anemia) or other early-onset ataxias (ataxia-oculomotor apraxia types 1 and 2). Only LCLs cells deficient in ATM, nibrin (NBS1), or Mre11 (ATLD) proteins differed from WT (Fig. 5B–D). This might have been anticipated, since both nibrin and Mre11 proteins play prominent roles in recruiting ATM to DNA sites of double strand breaks. The nibrin- and Mre11-deficient cells showed shifts comparable to that of A-T heterozygotes; however, neither resembled the FC-pSMC1 pattern of an A-T homozygote. These findings would not present a clinical testing problem, since Mre11 and NBS patients have phenotypes that are easily distinguishable from the normal phenotype of A-T heterozygotes. Cells from patients with DNA ligase IV deficiency, AOA1 (aprataxin deficiency), AOA2 (senataxin deficiency), and FA D2 (FANCD2 deficiency) showed WT patterns (Fig. 5E–H). Although ligase IV cells are radiosensitive, ligase IV protein functions pri-
mainly in the nonhomologous end joining pathway, in a manner that is not directly dependent on ATM kinase activity. On the basis of this very limited sampling, FC-pSMC1 appears to be a useful test for distinguishing A-T patients from other genomic instability disorders. An immunoblot (Fig. 6) using nuclear extracts from some of the same LCLs gave comparable SMC1-S966 phosphorylation results; however, the degree of correlation between FC-pSMC1 and immunoblotting will require further evaluation.

Discussion

Presently, diagnostic laboratory testing for A-T involves establishing LCLs to perform immunoblotting (9) and CSA (8). Immunoblotting of nuclear lysates from LCLs is performed to determine the presence or absence of ATM protein, which is absent in >99% of A-T patients (9). Although the current diagnostic protocol is extremely sensitive, it is labor intensive and has a long turnaround time. Recently, we developed an immunoassay (ATM-ELISA) to measure ATM protein concentrations directly from whole blood (10); however, this assay does not identify the rare A-T patients with kinase-dead ATM protein and is limited by the recurring requirement for a purified ATM protein standard (11). In addition, we were unable to reliably detect ATM protein on 3-day-old blood samples with this method, for reasons that are as yet undetermined. Prompted by these limitations, we evaluated the FC-pSMC1 assay for rapid diagnostic evaluation of suspected A-T. On the basis of the data in Fig. 1C, we believe that FC-pSMC1 alone provides a rapid, reliable, and cost-effective means of diagnosing A-T. This would reduce the turnaround time from approximately 3 months to <1 day. The cost-effectiveness of follow-up testing, such as by immunoblotting, ATM ELISA, CSA, and mutation detection, could then be reevaluated.

As noted above, A-T heterozygotes are susceptible to certain forms of cancer (14–25) and potentially to coronary disease (27). They constitute at least 1% of the general population, i.e., approximately 3 million Americans (20). Despite this, a reliable assay for identifying A-T carriers has been elusive. Previous studies attempted to identify A-T carriers on the basis of radiosensitivity testing using CSAs under hypoxic conditions (14). Once the ATM gene was localized to chromosome 11q23 (28), we were able to reanalyze our colony survival data on a 61-member Amish pedigree; however, once again, heterozygote identification was only 85%–90% accurate (15). The data presented herein strongly suggest that the FC-pSMC1 assay may provide the first reliable assay for carrier detection. At a minimum, it could be used to prescreen potential A-T carriers before confirmation by more costly and complex DNA sequencing.

Is SMC1 a good phosphorylation target for diagnostic A-T testing? It has been suggested that H2AX may be a better target (29–31) for detecting radiosensitivity. H2AX is rapidly phosphorylated (γ H2AX) in response to DNA double-strand breaks and is thought to recruit repair proteins to these sites. Our goal was targeted more toward diagnosing A-T than general radiosensitivity. In our hands, we were not satisfied with
the reliability of H2AX phosphorylation as a surrogate marker for A-T. Furthermore, many substrates of ATM, such as H2AX, Chk2, Chk1, and P53, are redundantly phosphorylated by other serine/threonine kinases involved in DNA damage repair (32, 33), sometimes targeting the same serine residues. For example, ATR (ataxia-telangiectasia Rad 3-related protein) and DNA-PK (DNA-dependent protein kinase) redundantly phosphorylate H2AX at Ser 139 or P53 at Ser 15, following DNA damage by ionizing radiation. This redundancy clouds diagnostic interpretations. As of this writing, SMC1 is thought to be phosphorylated directly by ATM following IR damage, and our results support this conclusion. For these reasons, we chose SMC1 over H2AX as an ATM-dependent target. On the other hand, that is not to say that using H2AX as a target might not be useful as an adjunctive assay for evaluating DNA damage or genomic instability. Indeed, even by adding a second time point (e.g., 24 h post-IR) for assessing SMC1 phosphorylation, much additional information might be gained regarding the efficiency of DNA repair pathways.

It should also be noted that the FC-pSMC1 assay cannot be used to reliably diagnose all radiosensitive
patients. For example, cells from Fanconi anemia FANC D2-deficient patients are radiosensitive despite showing normal ATM-dependent phosphorylation of SMCI by immunoblotting (34) and by FC-pSMCI (Fig. 3). It is also unlikely that measuring the phosphorylation of H2AX alone will provide a reliable test for predicting radiosensitivity. No doubt a panel of assays will be necessary to achieve this goal.

Using PBMCs from unknowns, obligate A-T heterozygotes (i.e., parents of A-T patients), and laboratory-confirmed A-T homozygotes, we were able to reliably discriminate between the 3 subsets, even using blood samples that were 3 days old. Only 1–2 mL of blood was required. Measurement using FC-pSMCI should be normalized against a known healthy daily control and the \( \Delta \)GMFI expressed as a percentage of the daily control. For the present, samples falling within the A-T heterozygote range should be confirmed by sequencing the ATM gene for mutations. In some instances, the increase in \( \Delta \)GMFI of unknown cells was larger than the GMFI of the daily control (e.g., see Fig. 3). Those circumstances are most likely due to the sensitivity of flow cytometry and should be considered as “equal to or greater than” control and interpreted as within normal limits. These accounted for most of the intraday variance (online Supplemental Table 2). This variability could be further reduced by using more than one daily control, as is done for other cellular assays; however, this may prove overly laborious since a high FC-pSMCI response is still within normal limits and has no clinical significance.

The FC-pSMCI assay can be performed in several hours and can be performed in most laboratories, using a flow cytometer. Bleomycin can be substituted if a cell irradiator is not available (26). The assay may be useful for prescreening large study populations to determine which samples should be sequenced to determine A-T heterozygosity, thereby circumventing the need to sequence all study subjects. Last, the functional consequences of missense DNA variants in the ATM gene can also be evaluated by FC-pSMCI testing, and this can be further applied to testing of stably transfected mutagenized A-T host cells (35).

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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