Early diagnosis of ataxia-telangiectasia using radiosensitivity testing

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Objectives: To utilize radiosensitivity testing to improve early diagnosis of patients with ataxia-telangiectasia (A-T).

Study design: We established normal ranges for the colony survival assay (CSA) by testing cells from 104 patients with typical A-T, 29 phenotypic normal patients, and 19 A-T heterozygotes. We also analyzed 61 samples from patients suspected of having A-T and 25 patients with related disorders to compare the CSA with other criteria in the diagnosis of A-T.

Results: When cells were irradiated with 1.0 Gy, the mean survival fraction (μSF ± 1 SD) for patients with A-T was 13.1% ± 7.2% compared with 50.1% ± 13.5% for healthy control patients. These data served to define a diagnostic range for the CSA (ie, <21%), a normal range (>36%), and a nondiagnostic intermediate range of 21% to 36%. The mutations of patients with A-T with intermediate radiosensitivity tended to cluster around the functional domains of the ATM gene.

Conclusions: The CSA is a useful adjunctive test for confirming an early clinical diagnosis of A-T. However, CSA is also abnormal in other chromosomal instability and immunodeficiency disorders. (J Pediatr 2002;140:724-31)

The diagnosis of ataxia-telangiectasia (A-T) is primarily clinical and is based on a progressive cerebellar ataxia of early onset.[1] With time, ocular apraxia can be demonstrated in almost all patients, oculocutaneous telangiectasias appear, and dysarthria becomes apparent. In young children, however, this diagnosis is often difficult to establish, which can delay genetic counseling and family planning. Laboratory findings include (1) elevated serum alphafetoprotein (AFP), (2) immunologic deficiencies, (3) characteristic chromosomal aberrations, (4) decreased or absent intracellular ataxia-telangiectasia mutated (ATM) protein levels by Western blotting, (5) deficient ATM phosphorylation of p53 at serine 15, (6) ATM mutations, and (7) in vitro radiosensitivity. Some of these tests are not available clinically. In vitro radiosensitivity testing, for example, has been done only on skin fibroblasts and in research laboratories; this necessitates a skin biopsy, and fibroblasts from patients with A-T grow poorly in tissue culture. Chromosome breakage studies after irradiation or bleomycin exposure have not proved sufficiently reliable for diagnostic testing.

We established an assay for evaluating in vitro radiosensitivity on clinical blood samples, the colony survival assay (CSA), in 1993.[2] Since that time, CSA results for 104 patients with typical A-T with known ATM mutations were accumulated; these results were analyzed and compared with other diagnostic variables such as serum AFP levels, ATM protein levels, and clinical progression. CSA is a reliable adjunctive diagnostic method for A-T; it may be especially useful in young families seeking genetic counseling and prenatal diagnosis, which must be based on a correctly diagnosed prior affected member.

AFP	Alphafetoprotein
A-T	Ataxia-telangiectasia
ATM	Ataxia-telanigectasia mutated
CFE	Colony-forming efficiency
CSA	Colony survival assay
LCL	Lymphoblastoid cell line
NBS	Nijmegen breakage syndrome
SF	Survival fraction
SSCP	Single-stranded conformational polymorphism

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METHODS

For the CSA,[2] 5 mL to 10 mL of heparinized blood was collected from patients suspected of having A-T, under institutional review board–approved protocols. Peripheral blood lymphocytes were isolated on a Ficoll-Hypaque gradient (Pharmacia, Peapack, NJ) and transformed with the Epstein-Barr virus. The transformed lymphoblastoid cells (lymphoblastoid cell line, LCLs) were maintained in 15% fetal bovine serum (Hyclone, Logan, Utah) and 1% penicillin/streptomycin (Gibco BRL, Grand Island, NY) at 37°C and 5% CO_2. Cells were grown to log phase (1×106 cells/mL), then plated in duplicate 96-well plates at 200, 100, or 50 cells per well. One plate was exposed to 1.0 Gy radiation and the other was kept as a control. Subsequently, the cells were incubated for 10 to 13 days, at which time they were stained with MTT dye (tetrazolium-based colorimetric assay, Sigma, St Louis, Mo). After a 2- to 4-hour incubation, each well was checked under the microscope; viable cells were dark blue. The presence of a colony of >32 cells was scored as a positive well. Colony-forming efficiency (CFE) was calculated as CFE = (–ln F)/W, where F is the fraction of negative wells and W is the number of cells seeded per well. The survival fraction (SF) after irradiation was obtained by dividing CFE_i (the CFE of irradiated plate) by CFE_c (the CFE of control plate): $SF = (CFE_i/CFE_c) \times 100$.

Normal and bonafide A-T LCLs were included as negative and positive daily controls, respectively. Each patient's LCL was tested multiple times.

ATM mutations were detected by various screening methods, including protein truncation testing,[3] conformation sensitive gel electrophoresis,[4] single-stranded conformational polymorphism (SSCP) analysis,[5] denaturing high-performance liquid chromatography,[6] or multiconditional SSCP (DOVAM).[7] Ultimately, all mutations were identified by DNA sequencing.

Western analysis was carried out for ATM protein detection. LCLs were collected and prepared as lysates, as previously described.[8] The proteins were run on 6% SDS–polyacrylamide gel electrophoresis and transferred onto PVDF membrane (BioRad, Hercules, Calif). In early experiments, a monoclonal ATM antibody, 3E8 (courtesy of Dr Eva Lee), was used to detect the ATM protein. Later, various commercial ATM antibodies were used with equally reliable results. Normal controls were standardized as having 4+ protein.

Serum AFP was measured by Tandem E AFP (ELISA) assay, according to the manufacturer's recommendations. Serum samples were stored frozen until tested. Coefficients of variation were <10%. All assays were measured in duplicate. Ages of those tested were all >6 months. Body weights were all within the 10th to 75th percentile. Normal AFP levels were ≤10 ng/mL. The minimal detectable concentration of AFP is estimated to be ~2 ng/mL.

ATM kinase assays for phosphorylation of p53 at serine 15 were performed on 10×10^6 LCLs from each patient. Cells were harvested 15 minutes after irradiation with 2.0 Gy. Cells were suspended in a borate lysis buffer and sonicated to prepare whole cell lysates; 35 μg of protein was electrophoresed on a 7% denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were blocked overnight with 5% powdered milk at 4°C followed by an overnight incubation with an antibody against phosphorylated-serine 15 of p53 (Cell Signalling, Beverly, Mass). Blots were washed, incubated with an anti-rabbit horseradish peroxidase secondary antibody, washed again, and visualized by enhanced chemiluminescence (BioRad).

RESULTS

CSA: Normal and Diagnostic Ranges

Our goals were to first determine the normal and abnormal ranges for the CSA and to then evaluate its specificity and sensitivity. We blindly tested the radiosensitivity of 167 consecutive LCLs derived from individuals suspected of having A-T. Subsequent testing identified mutations in the ATM gene in 106 of these patients (95 with 2 defined mutations; 11 with 1 defined mutation). LCLs of 104 patients with typical A-T were more sensitive to radiation than were those of 29 healthy control patients, with mean SFs (μSF ± 1 SD) of 13.1% ± 7.2% and 50.1% ± 13.5%, respectively ($P < .01$) (Fig 1). The mean SF for A-T genotypic heterozygotes (ie, genetically characterized) was 44.8% ± 13.8% (n = 19) and was indistinguishable from normal levels under the experimental conditions we selected. Further analyses (based as well on review of Western blots, ATM kinase activity, AFP levels, and ATM mutations) determined that the 167 samples also included 2 siblings with clinically atypical A-T and 61 patients with other neurologic disorders (mostly undiagnosed).

CSA: Specificity

If the specificity is evaluated by testing only children with an early-onset ataxia, the only confounding diagnosis would be with MRE 11–deficient patients.[9] To further evaluate the specificity of the CSA, LCLs from 25 patients with related disorders (mostly chromosomal instability syndromes) were tested.

RADIONORMAL. LCLs from 5 patients with Friedreich ataxia (genetically proven) were not radiosensitive. Two patients with Rothmund-Thomson syndrome (courtesy of Lisa Wang), a helicase deficiency, were also normal. LCLs from 1 patient with typical A-T and 2 with atypical A-T were not radiosensitive when tested at 1.0 Gy (ie, false-negative) and are further detailed below. The diagnoses of the other 61 patients with normal CSA responses are still largely undetermined. These patients encompassed a wide variety of neurologic signs and symptoms, such as mental and/or motor retardation and seizures. LCLs from 2 patients

Table I. Diagnostic data for patients with low normal and intermediate CSA results at 1.0 Gy*

Name code	CSA (1 Gy)	AFP (μg/μL)	ATM protein§	P53 kinase activity	Mutation‡	
Low normal						
AT24RM	40%	71	Neg	Neg	a) 755 delGT(ter)	b) 755 delGT(ter)
AT82LA†	37%	726	Neg	Neg	a) 697del405(x8-9)	b) 5290delC(ter)
AT83LA†	37%	>300	Neg	Neg	a) 697del405(x8-9)	b) 5290delC(ter)
Intermediate						
WAR16	32%	88	Neg	Neg	a) 8766insT(ter)	b) 3848T >C(miss)
TAT47	29%	51	++	Neg	a) 6188G >A(miss)	b) 6188G >A(miss)
SPAT11–3	29%	426	Neg	Neg	a) 8977C > T(ter)	b) 9170delGA(ter)
AT57LA	27%	31	Neg	Neg	a) IVS40+1126A > G	b) 5932G >T(ter)
AT12LA	25%	77	Neg	Neg	a) IVS40+1126A > G	b) (del exon 54)
AT143LA	23%		+	Intermediate	a) 875C > T(miss)	b) 8494C >T(miss)
AT42LA	22%	176	Neg	Neg	a) 103C > T(ter)	b) 8105T >G(miss)

*Dose-response curves (Fig 2) demonstrated intermediate radiosensitivity for the first 3 patients as well.
†Sibs with atypical A-T phenotype walked unassisted until 30 years of age.
‡*ter*, terminating mutation; *miss*, missense mutation; *Neg*, negative.
§Normal ATM protein level = ++++.

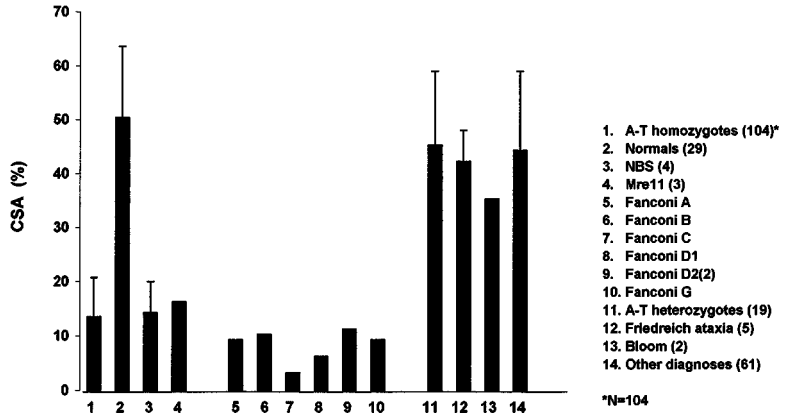

Fig 1. Mean colony survival fractions (μSF ± SD) after 1.0-Gy irradiation to LCLs of A-T homozygotes, Normals, A-T heterozygotes, and AT-related disorders. Numbers in parentheses indicate number of LCLs tested from different patients.

with Bloom syndrome (courtesy of James German), also a helicase deficiency had slightly increased radiosensitivity (Fig 1).

RADIOSENSITIVE. LCLs from 4 patients with Nijmegen breakage syndrome (NBS) had the same degree of radiosensitivity as A-T cells (μSF = 13.7% ± 9.0%) (Fig 1). LCLs from 7 patients with Fanconi's anemia (groups A, B, C, D1, D2, and G) (courtesy of Manuel Buchwald, Hans Joenje, and Alan D'Andrea) were also as radiosensitive (Fig 1). LCLs (courtesy of Malcolm Taylor) from 3 patients with Mre11 deficiency[9] were tested: the mean SF for an LCL from the first family was 7%; the μSF for the second family, 18% and 9%.

CSA: Intermediate Sensitivity, a Nondiagnostic Range

An intermediate range of 21% to 36% could be defined by using 1.0 SD above the A-T mean of 13.1% ± 7.2% and 1 SD below the normal mean of 50.1% ± 13.5% (or 23% to 28%, using 2 SD); 34 of the 238 individuals fell within this nondiagnostic, intermediate response range. Subsequent analyses of this group (based on the results of ATM mutation testing, Western blotting, and ATM kinase activity) identified 7 as patients with A-T (7 of 104 patients with typical A-T = 7%). All were retested with radiation exposures of 0.5, 1.0, 1.5, and 2.0 Gy. At 2.0 Gy, all patients with A-T were clearly radiosensitive (Fig 2). Further details on the 7 patients with A-T with intermediate radiosensitivity are shown in Table I.

Also manifesting intermediate CSA responses were a patient with NBS (a homozygous 657del5 NBS1 mutation), 5 A-T heterozygotes (determined by sequencing), 16 individuals with other diseases (as described above), and 5 phenotypically normal individuals. A CSA dose-response curve for the latter 5 normal individuals remained in the intermediate or low-normal range (data not shown). Three of these control patients were genotypically characterized as not being A-T heterozygotes (ie, they did not carry the affected haplotype within an affected family). In sum, by using a 1.0-Gy exposure, 5 of 29 normal individuals (17%) had intermediate CSA levels (these results are included in the mean SF for "normal patients" cited above); none of these LCLs scored

below 21% (Fig 2), the diagnostic cutoff point for abnormal.

One of the 104 patients with typical A-T (AT24RM) was in the "low normal" (or false-negative) range when tested at 1.0 Gy (29%, 48%, 43%). However, at 2.0 Gy, the LCLs were clearly radiosensitive (Fig 2). This patient was from a consanguineous family and had a homozygous ATM mutation[10] (Table I). Two brothers (AT82LA and AT83LA) with atypical A-T phenotypes also had "low normal" CSA results at 1.0 Gy but were radiosensitive at 1.5 Gy and 2.0 Gy (Fig 2). Both sibs were able to walk unassisted (nonetheless, with ataxia) until 30 years of age. In retrospect, by using 2.0 Gy instead of 1.0 Gy, the sensitivity of the CSA can be further improved, although patients with intermediate responses would then be more difficult to identify. We conclude that whereas only 1 in 104 false-positives were observed by using 1.0 Gy (ie, sensitivity = >99%), 7 additional patients with A-T fell outside the diagnostic range (ie, sensitivity = >92%).

Serum AFP Levels

We compared the CSA results with serum AFP levels for 64 individuals, including 37 patients with A-T; 35 (95%) patients with A-T had elevated AFP levels, whereas 2 (5%) had normal AFP levels. Twelve of the 64 individuals had intermediate CSA levels (21% to 36%): 6 had A-T (all with elevated AFPs), 1 had NBS (normal AFP), and 5 remained undiagnosed. Four individuals with elevated AFP levels could not be confirmed as A-T by all other criteria examined (ages 3, 5, 20, and 47 years); CSA results were normal. Sixteen of the 64 individuals had normal AFP, normal CSA levels, normal intracellular ATM protein levels, and were assumed not to have A-T.

Intracellular ATM Protein Levels

Intracellular ATM protein levels were compared with CSA results on LCLs

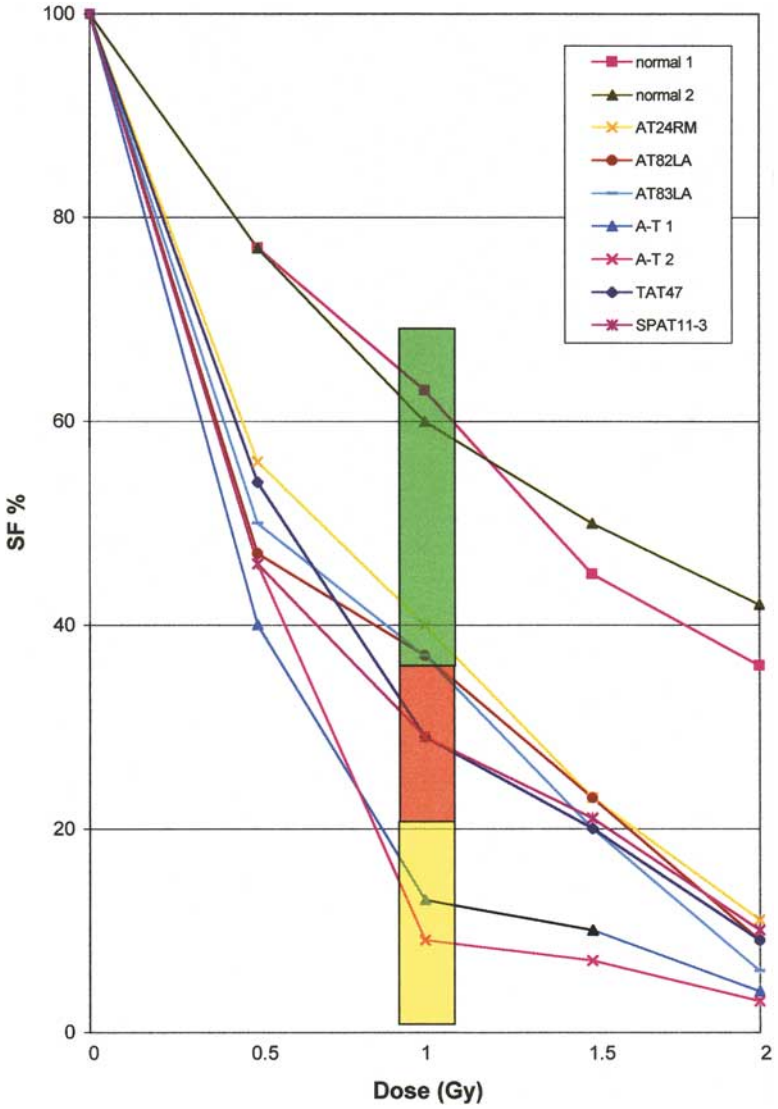

Fig 2. Dose-response curves for patients with intermediate and "low normal" CSA results when previously tested at 1.0 Gy. Normal range, green; intermediate range, red; radiosensitive range, yellow. "Normal" and "A-T" in this figure refer to positive and negative preselected control LCLs used for these experiments; however, the intermediate responders are also patients with A-T.

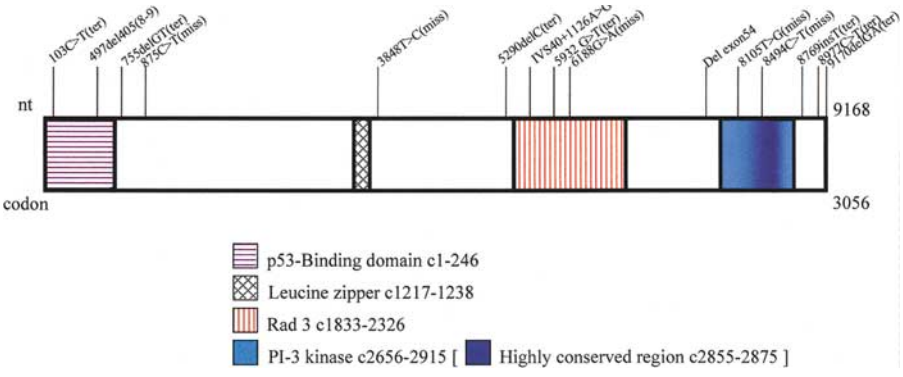

Fig 3. ATM mutations associated with intermediate and low-normal radiosensitivity. Note clustering into three domains. Additional data on these patients are shown in Table I.

from 68 patients with A-T, 7 normal patients, and 2 A-T heterozygotes. Of 62 LCLs with CSA <21%, 59 had no detectable ATM protein (95%) and were considered to have A-T (mutations were subsequently identified on all of these); 3 LCLs had 1 to 2+ intracellular ATM protein (5%). Of 10 LCLs with intermediate or "low normal" CSA results, 8 had no detectable protein (80%) and 2 had 1 to 2+ intracellular ATM protein (Table I). Thus, whereas intracellular ATM protein levels correlated well with CSA results, some discrepancies were noted that proved to be of diagnostic value.

Mutations

ATM mutations were identified in 106 patients with A-T. Many of these mutations have been described elsewhere and are distributed randomly across the ATM gene.[3,5,7,11] However, when the mutations of the patients with A-T with intermediate sensitivity were plotted onto a domain map of the ATM gene/protein, most of them fell strikingly within either the p53-binding, leucine zipper, RAD3 homology, or PI-3 kinase domains (Fig 3). The two sibs with atypical A-T had two splicing mutations, one within the p53-binding domain, the other just proximal to the most conserved region of the RAD3 homology domain (Table I and Fig 3). In the 5 patients described above with detectable intracellular ATM protein, 8 mutations were identified; 5 were missense mutations (63%). This contrasts sharply with the 15% frequency of missense mutations in the ATM Mutation Database (http://www.vmresearch/atm.htm).

DISCUSSION

Radiosensitivity, a major hallmark of A-T,[1,12] was first noted after exposure of a patient to conventional doses of radiation therapy, which proved fatal.[13] In vitro radiosensitivity was subsequently described by Higurashi and Conen,[14] who demonstrated elevated levels of chromosomal aberrations in lymphocytes from patients with A-T, Bloom, and Fanconi syndromes after 1.0 Gy of irradiation. Taylor et al[15] expanded this observation by using A-T fibroblasts. Paterson et al[16] reported that some A-T fibroblasts had a reduced capacity to remove thymine glycol damage from DNA at high radiation doses. Coquerelle et al[17,18] found that rejoining of gamma radiation–induced DNA double-strand breaks was slower in fibroblasts from patients with A-T and Fanconi syndrome than in control patients. After irradiation, A-T cells are characterized by faulty G_1, S, and G_2/M checkpoints. They also exhibit radioresistant DNA synthesis. The inability of A-T cells to activate these checkpoints efficiently ultimately leads to accumulation of chromosomal damage in the G_2/M phase, where such cells would normally be targeted for apoptosis. An elevated rate of apoptosis after radiation has been reported for A-T cells of some cell lineages but not others. It has been suggested that intermediate levels of radiosensitivity may be of prognostic value for A-T homozygotes.[19]

Several studies have demonstrated an intermediate level of radiosensitivity in fibroblasts from A-T heterozygotes.[20-22] Paterson et al[21] and Tchirkov et al[22] reported that both A-T homozygotes and A-T heterozygotes showed significantly increased levels of radiation-induced chromosomal damage relative to that of normal control patients. However, the accuracy of identifying A-T heterozygotes by such methods does not exceed 85%.[23] We found that the CSA results for A-T heterozygotes did not differ significantly from the normal patients. Had we observed intermediate CSA levels for heterozygotes, this might have posed a confounding factor, because A-T heterozygotes are estimated to comprise 1% to 2% of the white population in the United States,[24] and this figure may actually be as high as 5% to 8%.[25] Weissberg et al[26] found no evidence for abnormal clinical radiosensitivity in two A-T heterozygotes receiving conventionally fractionated radiation therapy for breast or prostate cancer.

Patients with Mre11 deficiency[9,27] could possibly be mistaken for having mild A-T, which exemplifies the importance of establishing a correct diagnosis for the ataxia before prenatal testing by haplotyping. In our hands, the in vitro radiosensitivity in a patient from the first Mre11 deficiency family was not intermediate but was indistinguishable from that of a patient with typical A-T. Nonetheless, an experienced neurologist would note the slower neurologic deterioration of an Mre11-deficient patient.[28] Western blotting of cell lysates from such patients would further show normal intracellular ATM protein levels and absent or reduced levels of Mre11, nibrin, and Rad 50.[27] For patients with Friedreich ataxia, on the other hand, the late-onset ataxia could be distinguished from that of Mre11 deficiency by the finding of a normal CSA.

Patients with NBS (formerly called A-T Variant 1) are also radiosensitive and have immunodeficiencies, cancer, and chromosomal changes.[29] The ATM protein phosphorylates the NBS1 protein,[30] thereby accounting for the overlapping syndromes of A-T and NBS. AT_{Fresno} patients share the combined syndromes of A-T and NBS; they have ATM mutations, the NBS gene being normal.

The product of the ATM gene is a predominantly nuclear phosphoprotein, which functions primarily as a PI-3 kinase.[30,31] In response to double-strand breaks in DNA, ATM binds p53 and activates it by phosphorylating the serine at position 15. ATM also controls other phosphorylation, and dephosphorylation, changes.[32] ATM is a member of the large BRCA1-associated surveillance DNA repair complex: BASC.[33] ATM phosphorylates BRCA1, directly and indirectly.[34,35] Despite these new

insights into ATM functions, the mechanisms underlying the radiosensitivity of A-T LCLs in the CSA are not clearly understood. Generally, they involve the repair of double-strand DNA breaks. The Rad50/Mre11/nibrin DNA repair complex and its upstream and downstream partners must also be part of this mechanism, judging from the fact that cells from patients with NBS, Mre11,[36,37] and A-T are all radiosensitive. How the Fanconi proteins confer radiosensitivity in the CSA assay also remains unclear, although the phosphorylation of Fanconi's anemia D2 is ATM dependent.[38]

Only 4 functional domains or consensus sequences have as yet been convincingly described for the ATM gene[1]: p53-binding, a leucine zipper region, RAD3 homology, and PI3 kinase homology (Fig 3). Almost every patient with A-T with an intermediate CSA response carried a mutation(s) within either the PI-3 kinase, RAD3 homology, or p53-binding domains. This potential phenotype/genotype correlation stands in stark contrast to the distribution of >400 mutations across the entire gene in other patients with A-T [http://www.vmresearch.org/atm.htm]. Such a finding would have been more easily understood had these been patients of extreme rather than intermediate radiosensitivity. A further confounding factor is that most of the mutations in these intermediate sensitivity patients would result in truncated and unstable proteins; furthermore, Western blots of lysates from most of the same LCLs show no ATM protein (Table I). Thus, the genomic position of the mutations should be of little functional significance. The observation suggests that small amounts of ATM protein or truncated protein fragments are made in A-T cells and that these must interact with other proteins before they are degraded, perhaps in a dominant-negative manner.[25]

The laboratory workup for a clinically suspected diagnosis of A-T is summarized in Table II. Because the turnaround time for CSA is ~3 months, finding a faster and more efficient diagnostic method for A-T would be advantageous. One obvious alternative would be to measure intracellular ATM protein in peripheral blood lymphocytes. However, this currently involves othe pitfalls: (1) intracellular ATM protein levels of peripheral blood lymphocytes are very low and not reliably detected by Western blots of extracts derived from <10 mL of blood, and failure to detect the protein might result in a "false-negative" report, supporting a misdiagnosis; and (2) attempts to stimulate ATM expression with mitogens[19,39] might be confounded by the underlying poor response of lymphocytes from many A-T patients to mitogen stimulation.[40,41] Once an LCL has been established and 10×10^6 LCLs are available for preparation of a cell lysate, the Western blot requires only 2 days and has a diagnostic sensitivity of >85%.[8] On the other hand, the CSA requires only 1×10^5 LCLs and therefore can be performed earlier. The intermediate response range of 21% to 36% is nondiagnostic because although it includes 7% of patients with A-T, it also includes 17% of normal patients. By performing the CSA with 2.0-Gy exposure to cells, intermediate responses would be encountered far less frequently.

Table II. Laboratory confirmation of A-T

	Not informative (%)
1. Alphafetoprotein*	5
2. Karyotyping* (technically difficult with A-T cells	~10[1]
3. Radiosensitivity	
Radioresistant DNA synthesis—not available in clinical laboratories	<1?[1]
Colony survival assay*	<8
Chromatid breaks*	15[1]
4. Western blots* (for ATM protein in lysates of LCLs)	<5
5. Kinase activity (p53/Serine 15)(if ATM protein is present)	<1?
6. Mutation detection	
If known, from a prior affected	<1
Unknown—Protein truncation test to detect one allele (uses mRNA)	<20[1]
—SSCP, conformation sensitive gel electrophoresis, DOVAM, or REF	<10[1]
—Automated sequencing	<30[1]
—denaturing high-performance liquid chromatography (heteroduplex detection; misses homozygotes)	~15[1]
7. Haplotype family—genetic linkage to 11q23	25[1]

*Available in clinical laboratories.

CSA might also be used as an adjunctive diagnostic test in other chromosomal instability and immunodeficiency disorders, such as NBS, Mre11 deficiency, X-linked agammaglobulinemia,[2] severe combined immunodeficiency, and Fanconi's anemia. In patients with a history of untoward reactions to conventional doses of radiation therapy, CSA may also be used to study and identify new radiosensitive diseases such as the recently described ligase IV deficiency syndrome.[42]

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