Ataxia-Telangiectasia: Mild Neurological Presentation Despite Null *ATM* Mutation and Severe Cellular Phenotype

Neora Alterman,[1] Aviva Fattal-Valevski,[2] Lilach Moyal,[1] Thomas O. Crawford,[3] Howard M. Lederman,[4] Yael Ziv,[1] and Yosef Shiloh[1]*

[1]The David and Inez Myers Laboratory for Genetic Research, Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel
[2]The Institute for Child Development & Pediatric Neurology Unit, "Dana" Children's Hospital, Tel Aviv-Sourasky Medical Center, Tel Aviv, Israel
[3]Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland
[4]Division of Pediatric Allergy and Immunology, Johns Hopkins University School of Medicine, Baltimore, Maryland

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder characterized by progressive neurodegeneration, immunodeficiency, susceptibility to cancer, genomic instability, and sensitivity to ionizing radiation. A-T is caused by mutations that eliminate or inactivate the nuclear protein kinase ATM, the chief activator of the cellular response to double strand breaks (DSBs) in the DNA. Mild A-T is usually caused by *ATM* mutations that leave residual amounts of active ATM. We studied two siblings with mild A-T, as defined by clinical examination and a quantitative A-T neurological index. Surprisingly, no ATM was detected in the patients' cells, and sequence analysis revealed that they were homozygous for a truncating *ATM* mutation (5653delA) that is expected to lead to the classical, severe neurological presentation. Moreover, the cellular phenotype of these patients was indistinguishable from that of classical A-T: all the tested parameters of the DSB response were severely defective as in typical A-T. This analysis shows that the severity of the neurological component of A-T is determined not only by *ATM* mutations but also by other influences yet to be found.
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Key words: ataxia-telangiectasia (A-T); mild A-T; A-T-like disease; cerebellar degeneration; ATM; DNA damage response

INTRODUCTION

Ataxia-telangiectasia (A-T) is a multisystem, autosomal recessive disorder whose hallmarks are progressive neuronal degeneration, affecting primarily the cerebellar cortex and leading to severe neuromotor dysfunction; oculocutaneous telangiectasias; deficient humoral and cell-mediated immunity; predisposition to malignancies, mainly of lymphoreticular origin; growth retardation; elevated levels of serum α-fetoprotein and carcinoembryonic antigen; genomic instability expressed as high rate of chromosomal breaks and clonal translocations in lymphoid cells; and acute sensitivity to ionizing radiation [Cabana et al., 1998; Crawford et al., 2000; Chun and Gatti, 2004; Taylor and Byrd, 2005]. Cells from A-T patients exhibit marked sensitivity to the cytotoxic effect of ionizing radiations and radio-mimetic chemicals, which is caused by a profound defect in the cellular responses to double strand breaks (DSBs) in the DNA.

A-T is caused by mutations in the *ATM* gene [Savitsky et al., 1995a,b], which encodes the nuclear protein kinase ATM, the chief activator of the cellular

response to DSB induction [Shiloh, 2003]. The DNA damage response is a complex signaling network that is activated by DNA damage. DSBs are particularly effective in activating this system, which spans numerous cellular processes [Shiloh, 2003, 2006; Kitagawa and Kastan, 2005]. Notable damage response pathways include the cell cycle checkpoints that temporarily arrest cell cycle progression while DNA damage is assessed and processed, but numerous other pathways are modulated in the DNA damage response as well. ATM activates this network in response to DSBs by phosphorylating key proteins in damage response pathways.

The classical A-T phenotype is caused by homozygosity or compound heterozygosity for null *ATM* alleles, which truncate ATM or completely inactivate it via missense mutations [Gilad et al., 1996b; Broeks et al., 1998; Stankovic et al., 1998; Sandoval et al., 1999; Teraoka et al., 1999; Becker-Catania et al., 2000; Laake et al., 2000; Li and Swift, 2000; Saviozzi et al., 2003]. Since truncated ATM and other inactive forms of ATM are highly unstable, cells from classical A-T patients usually lack detectable ATM [Becker-Catania et al., 2000]. Milder forms of A-T, which are characterized by later onset or slower progression of signs and symptoms, are associated with mutations that leave residual amounts of functional ATM. These include presumed regulatory mutations that affect the rate of *ATM* transcription, certain missense mutations that only partially ablate ATM's activity, and leaky splicing mutations that allow the production of small amounts of correctly spliced transcripts [Gilad et al., 1998; Taylor and Byrd, 2005]. In all these mild cases reported to date, a minimal amount of full-length ATM was detected or the mutations identified were of the "mild" type [Taylor et al., 1987; McConville et al., 1996; Gilad et al., 1998; Stankovic et al., 1998; Becker-Catania et al., 2000; Stewart et al., 2001; Saviozzi et al., 2002; Chun et al., 2003; Chun and Gatti, 2004; Dork et al., 2004; Sutton et al., 2004].

Another genomic instability syndrome, A-T-like disease (A-TLD), is similar to mild A-T, having later age of onset and slower progression than classical A-T [Taylor et al., 2004]. A-TLD is caused by hypomorphic mutations in the *MRE11* gene [Stewart et al., 1999; Fernet et al., 2005]. The Mre11 protein is a nuclease that is part of the Mre11-Rad50-Nbs1 (MRN) complex, a DSB sensor [Petrini and Theunissen, 2004; Stracker et al., 2004; Paull and Lee, 2005]. The similarity between A-T and A-TLD reflects the requirement of the MRN complex for ATM activation [Carson et al., 2003; Uziel et al., 2003; Costanzo et al., 2004; Lee and Paull, 2005].

Here we present two siblings with clinically mild A-T despite homozygosity for a truncating *ATM* mutation that leads to complete ATM deficiency, severe cellular phenotype, and cerebellar atrophy typical of classical A-T. This genotype–phenotype combination indicates that the severity of the neurological phenotype in A-T may be dissociated from the extent of neuronal degeneration and the severity of the molecular defect. These findings are important to correct diagnosis of A-T, an understanding of the underlying neuronal pathophysiology, and the search for effective therapies for this disease.

PATIENTS

The patients were two brothers in a consanguineous family of Circassian origin (Fig. 1). Patient V_4 was the healthy product of a term pregnancy and reportedly developed normally until the age 6 years when he first manifested progressive gait unsteadiness and dysarthria. Physical examination at age 16 demonstrated conjunctival telangiectasia. Neurologic examination demonstrated bilateral endgaze-evoked nystagmus and oculomotor apraxia apparent in large gaze-shifts, mild dysarthria and hypomimea, end-point dysmetria, mild choreiform movements at rest enhanced by mental or physical activation, and hyporeflexia. Pertinent normal features include good strength and normal sensory thresholds for small and large fiber modalities. Although his gait was ataxic, he was able to walk independently with caution. These features were substantially less severe in all facets than were all 62 patients with classical A-T over the age of 16 years who have been evaluated neurologically (TOC) at the Johns Hopkins A-T Clinical Center. There was no history of recurrent sinopulmonary infections or aspirations. Laboratory test values (Table I) are notable for elevated levels of serum α-fetoprotein, diminished levels of immunoglobulins IgA, IgG2, and IgE, and increased levels of IgM and IgG1. Brain MRI showed moderate atrophy of the vermis and cerebellum (Fig. 2).

His brother, Patient V_6 is one of non-identical twins born at term, who also developed normally throughout childhood. Evaluation at 9.5 years of age demonstrated mild bilateral conjunctival telangiectasia and neurologic examination revealed normal gait and ability to hop on one foot, no nystagmus, dysmetria or choreoathetosis, but some tendency to
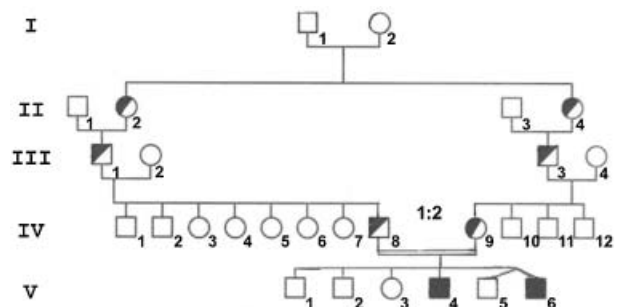

Fig. 1. Pedigree of family described in this study.

TABLE I. Laboratory Tests of the Described Patients

Laboratory test	Patient V_4	Patient V_6	Normal values
α-fetoprotein (ng/ml)	201.5	92.5	0–15
Carcinoembryonic antigen (ng/ml)	0.4	0.8	0–5
Total IgG (g/L)	15.4	10.2	7–16
IgA (g/L)	<0.234	1.55	0.7–4
IgM (g/L)	2.38	1.37	0.4–2.3
IgG1 (g/L)	12.53	7.45	3.15–8.55
IgG2 (g/L)	0.25	1.02	0.64–4.95
IgG3 (g/L)	1.10	0.37	0.23–1.96
IgG4 (g/L)	0.97	<0.01	0.11–1.57
IgE (U/ml)	<3	Not tested	20–100
Lymphocytes total (10^3/μl)	1.3	1.6	1.2–3.0

sway while sitting and difficulty standing in one position with feet together, difficulty with tandem gait, mild dysarthria and tendency for drooling, difficulty with fast alternating movements (disdiadochokinesis), and handwriting. Overall, qualitative assessment suggested a substantially less impaired neurologic phenotype, though typical in character, than that of peers with classic A-T. There was no history of sinopulmonary infections. Abnormal laboratory tests included elevated serum α-fetoprotein and low levels of IgG4 (Table I). Brain MRI showed enlargement of the 4th ventricle and atrophy of the vermis (Fig. 2).

For both boys, qualitative features of the neurologic examination are highly characteristic of A-T. However, the severity of neurologic impairment was dramatically milder than average. Quantitative neurological assessment according to the multi-dimensional index of A-T neurologic impairment [Lewis et al., 1999; Crawford et al., 2000] demonstrated that patient V_4 scored 4.95 SDs (value 66), and patient V_6 4.31 SDs (value 91), above the mean score for age in the large Johns Hopkins A-T Clinical Center referral population of patients with A-T (Fig. 3). Patients who score greater than two SDs above the mean for age are classified as having mild A-T according to this
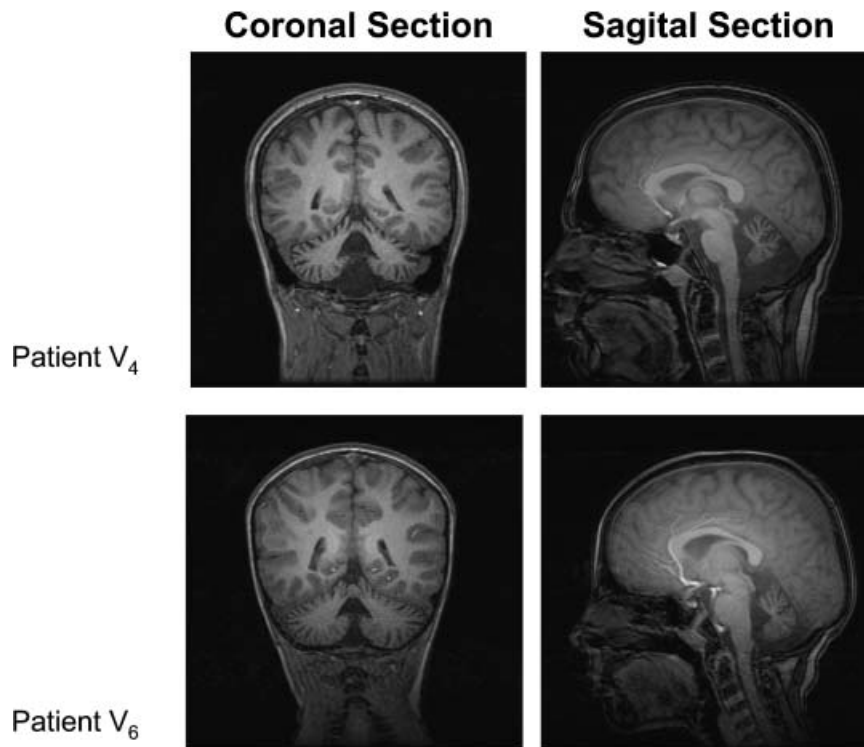

FIG. 2. Magnetic resonance imaging (MRI) images of the patients. Coronal and sagital section of T1-weighted MRI brain images are shown. Patient V_4 exhibits severe atrophy of the vermis and cerebellar hemispheres. Patient V_6 exhibits vermis atrophy and enlargement of the 4th ventricle.
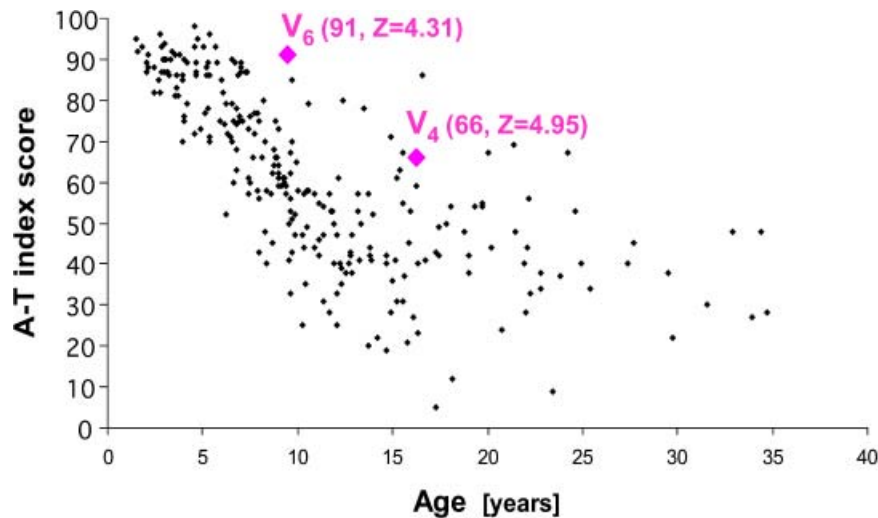
Fig. 3. A-T neurological index values [Crawford et al., 2000] as a function of patient age. A-T index values obtained for 255 examinations of 191 A-T patients appear in black. The A-T index values and Z scores (standard deviation from mean for age) of the two described patients appear in pink. The Z scores (SD) of the V_4 and V_6 patients are more than four standard deviations above the mean for their age. These indices indicate that the HH patients exhibit mild neurological symptoms compared to same-age A-T patients. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

index, which averages 10 statistically independent ordinal scales with 100 normal and 0 maximally impaired.

MATERIALS AND METHODS

Cell Culture and Treatment with DNA Damaging Agents

Wild-type lymphoblastoid cell lines C3ABR (from Martin Lavin, Queensland Institute of Medical Research, Brisbane, Australia), A-T lymphoblasts, AT24RM and AT59RM (from Luciana Chessa, Universitá La Sapienza, Rome, Italy), and L3 (from an A-T patient of Jewish–Moroccan extraction) [Gilad et al., 1996a] were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS). Lymphoblastoid cell lines were established from the described patients (cell lines YS3 from V_6 and YS4 from V_4) using EBV-mediated transformation, and grown similarly. Wild-type skin fibroblasts (F-2001) and A-T fibroblasts (F-2071 and F-2084) were grown in DMEM with 15% FBS. Skin fibroblasts from the patients (F-2111 from V_6 and F-2112 from V_4) were established from skin biopsies and grown in the same medium and immortalized by transducing the cells with a retroviral vector expressing the catalytic subunit of human telomerase (hTert) (obtained from Tej K. Pandita, Washington University, Saint Louis, MO) as described previously [Wood et al., 2001]. These cell lines, F-2111/hTert from patient V_6 and F-2112/hTert from patient V_4, as well as wild-type hTert fibroblasts HFF, A-T/hTert fibroblasts GM5823 (both from Tej K. Pandita), and hTert fibroblasts from an A-TLD patient, A-TLD2 (obtained from A. Malcolm Taylor, University of Birmingham, England

as primary fibroblasts and immortalized in our laboratory) [Uziel et al., 2003], were grown in *DMEM* medium supplemented with 20% FBS. All cells were grown at 37°C in humidified atmosphere with 5% CO_2. For DSB induction, cells were treated with various doses of the radiomimetic drug neocarzinostatin (NCS; Sigma–Aldrich Co., St. Louis, MO), or with ionizing radiation, using a 160HF irradiator (Philips, Hamburg, Germany).

Mutation Analysis

Total RNA was extracted from lymphoblastoid cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was carried out on 2.5 µg of RNA, in the presence of an oligo(dT) primer (Promega, Madison, WI) and SuperScript II Reverse Transcriptase (Gibco BRL Life Technologies, Grand Island, NY), in 10 µl reactions containing 2 units/µl of ribonuclease inhibitor (Sigma–Aldrich Co.), 10 mM dithiothreitol (Gibco BRL), and 0.5 mM dNTPs (Sigma–Aldrich Co.). The reaction products were used as templates for PCR based on eight primer pairs that amplify overlapping segments together spanning the entire *ATM* open reading frame. Reactions were carried out in 50 µl containing 2U Expand High Fidelity PCR Mix (Roche Applied Science, Indianapolis, IN), 200 µM dNTPs, 0.5 µM of each primer, and one-tenth of the RT-PCR products. The products were purified using the QIAquick gel extraction kit (Qiagen) and sequenced at Tel Aviv University, Sequencing Core.

Western Blotting Analysis and Antibodies

Cellular extracts were obtained by lysing the cells in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, Igepal 1% v/v (Nonident P-40 analogue, Sigma–Aldrich Co.), 1 mM

EDTA pH 8.0 supplemented with a protease and phosphatase inhibitors mixture. Cell pellets were suspended in lysis buffer and rotated at 4°C for 30 min, supernatants were collected after centrifugation at 21,000g for 20 min, and protein concentration was determined using the Bradford method (BioRad, Hercules, CA). 100–150 μg of cellular extracts were separated by SDS-PAGE and transferred overnight at 4°C onto a PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked for 40 min in 5% dry milk in Tween–Tris buffered saline (TTBS) and incubated overnight at 4°C with a primary antibody diluted in TTBS containing 1% bovine serum albumin. The following primary antibodies were used: anti-ATM monoclonal antibody MAT3-4G10/8 raised in our laboratory; anti-pSer957 of SMC1 (Novus Biologicals, Inc., Littleton, CO); anti-pSer824 of KAP-1 (Bethyl Laboratories, Inc., Montgomery, TX); anti-pSer15 of p53 and anti-pThr68 of Chk2 (Cell Signaling Technology, Danvers, MA); anti-β-tubulin (Sigma–Aldrich Co.). Secondary antibodies were goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP (Jackson ImmunoResearchLaboratories, West Grove, PA). Signals were visualized using enhanced chemiluminescence (Super Signal System, Pierce Chemical, Rockford, IL).

Cellular Sensitivity to Ionizing Radiation

For clonogenic growth assay, primary fibroblasts at late logarithmic stage were irradiated with varying doses of IR, and replated at densities of 100–10,000 cells per 60 mm plate. The cultures were incubated for 15–17 days in F-10 HAM medium supplemented with 20% FBS, fixed, and stained with 2% crystal violet in 50% ethanol. Colonies of at least 50 cells were counted. Surviving fraction for each dose was calculated and survival curves constructed.

Cell Cycle Analysis

Lymphoblastoid cell lines at logarithmic stage were irradiated with 2 Gy of X-rays. Aliquots of the cells were collected 12, 24, and 36 hrs post-irradiation; the cells were washed, suspended in 100 μl of cold PBS, and fixed by slow dripping into cold 70% ethanol. The cell suspensions were kept at −20°C, then spun at 200g, washed twice, and incubated in PBS on ice for 1 hr. After re-hydration, samples were spun again and pellets resuspended in 500 μl of PBS containing 25 μg/ml propidium iodide (Sigma–Aldrich Co.) and DNAse-free RNase (Roche). DNA content of 10,000 cells for each sample was analyzed by flow cytometry (FACSort, Becton Dickinson, San Jose, CA), and the percentage of cells in each phase of the cell cycle was determined using the ModFit program (Verity Software House, Inc., Topsham, ME). Cell cycle checkpoint activation was assessed by calculating the ratio between cells in G2 + mitosis

and cells in G1 ("G2/G1 ratio"). Statistical analysis was conducted using the Student's t-test.

RESULTS

Absence of ATM Protein, and a Classical A-T Mutation

The clinical phenotype of the described patients was reminiscent of mild A-T or possibly A-TLD. Mild A-T has low levels of full-length ATM and A-TLD patients have normal ATM levels and abnormal amounts of Mre11 protein. Unexpectedly, ATM was not detected in cells from either patient and Mre11 levels were normal—a typical laboratory presentation of classical A-T (Fig. 4A). The level of another member of the MRN complex, Nbs1 was also normal, and so were the levels of other members of the ATM-related protein kinase family, ATR, hSMG-1, and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) [Shiloh, 2003; Abraham, 2004; Bakkenist and Kastan, 2004] (not shown). Sequence analysis of *ATM*'s transcript (not shown) identified a deletion of one of two adjacent adenine
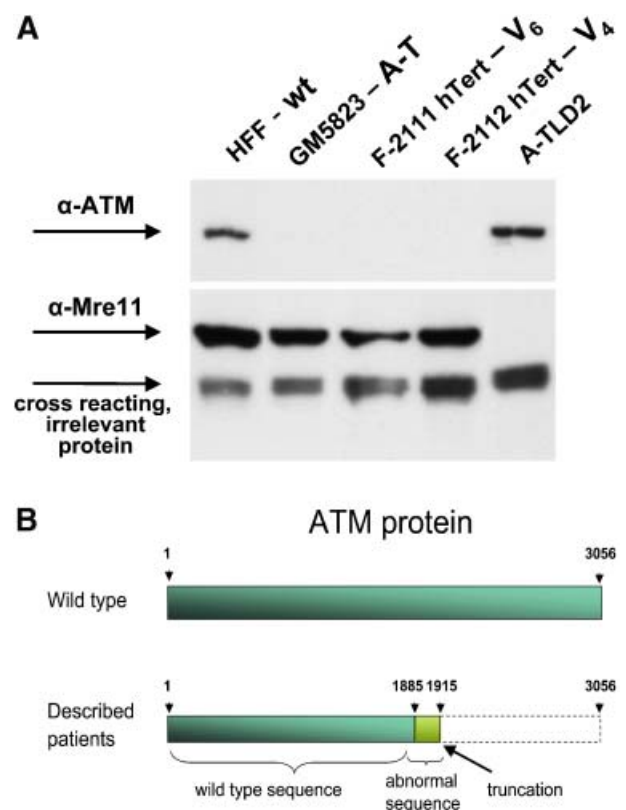

FIG. 4. **A**. Western blotting analysis of the ATM and Mre11 proteins in hTert-immortalized fibroblasts of patients V$_4$ and V$_6$ and controls. A cross-reacting band serves as a loading control. Note the absence of ATM, and normal Mre11 levels in the HH patients, similar to a patient with classical A-T. **B**. Expected effect of the 5653delA mutation on ATM protein: a frameshift at position 1885 leading to truncation at position 1915. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

residues at positions 5652 and 5653 of ATM's open reading frame, hereafter designated 5653delA. Genomic sequence analysis indicated that both patients were homozygous for this mutation, which is localized to exon 39 of the *ATM* gene, and their parents were carriers. The mutation, which had not been previously identified in other A-T patients, is expected to cause a frameshift and subsequent truncation of the ATM protein at position 1915 (Fig. 4B), eliminating ATM's active site from the translated protein. Since truncated ATM is usually unstable, A-T cells with such mutation are expected to completely lack ATM protein [Becker-Catania et al., 2000].

Classical A-T Cellular Phenotype

The discrepancy between the patients' mild clinical phenotype and the nature of their *ATM* mutation led us to examine their cellular phenotype. Cells from patients with classical A-T exhibit a profound defect in the DSB response due to the loss of the primary activator of this response, the ATM protein. Hallmarks of this phenotype are acute radiosensitivity and marked attenuation of ATM-mediated responses, such as activation of the cell cycle checkpoints and phosphorylation of ATM's downstream targets [Shiloh, 2003; Chun and Gatti, 2004]. We asked whether the cellular phenotype of the patients matched the severity of their genetic defect or the mild nature of their clinical phenotype.

Significantly, the severity of the defect in the DSB response in the described patients was indistinguishable from that of classical A-T. Clonogenic survival curves constructed following treatment with increasing X-ray doses showed that the described patients' cells were as radiosensitive as those of classical A-T patients (see the online Fig. 5A at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html). Similarly, activation of the cell cycle checkpoints in response to IR treatment was defective to a similar extent in cells from our patients and classical A-T cells (see the online Fig. 5B at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html). Finally, the readouts of ATM activity—phosphorylations of several ATM substrates following DSB induction—were diminished to similar extent in the described patients and A-T controls (see the online Fig. 5C at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html). We concluded that the defect in the DSB response in these patients was indistinguishable from that of classical A-T patients as expected based on their *ATM* mutation and the loss of ATM from their cells.

DISCUSSION

Mild A-T has been documented to date only in patients with residual levels of functional ATM and regulatory, missense, or leaky splicing *ATM* mutations [Taylor et al., 1987; McConville et al., 1996; Gilad et al., 1998; Stankovic et al., 1998; Stewart et al., 2001; Saviozzi et al., 2002; Chun et al., 2003; Chun and Gatti, 2004; Dork et al., 2004; Sutton et al., 2004]. We show here for the first time a mild A-T clinical phenotype despite the presence of homozygous classical A-T mutation that leads to complete loss of ATM and the severe, classical cellular phenotype.

The degree of cerebellar degeneration in both of the described patients matched their molecular defect but was unexpected in view of their mild neurologic phenotype. Phenotypic variability among classical A-T patients has been well documented based on multiple dimensions of their neurodegeneration [Crawford et al., 2000], but these patients manifest a departure from this variability by exhibiting mild expression of all the neurologic features known to be impaired in classical A-T. The reason for this discrepancy between genotype and phenotype is unknown.

One possible explanation for the genotype–phenotype discrepancy in these patients is the possible effect of modifier genes on the clinical outcome of A-T mutations. To date, one modifier of the effect of ATM loss has been identified—the Rad50S allele of the gene encoding the Rad50 component of the MRN complex [Bender et al., 2002; Usui et al., 2006]. The Rad50S allele partly compensates for the loss of ATM in the mouse, alleviating senescence, radiosensitivity and tumor formation, hallmarks of ATM-deficient mice [Morales et al., 2005]. In the mouse, this modifier acts at the level of the DSB response and is probably hypermorphic for the ATM-mediated DNA damage response [Bender et al., 2002; Usui et al., 2006]. The presumed modifier in our patients is probably different and exerts its effect at the level of central nervous system function rather than the DNA damage response. The rarity of such patients probably indicates that the highly influential alleles of such modifier genes are rare. It is interesting to note in this regard that this is the first documented A-T family in the Circassian community.

The presence of significant cerebellar atrophy, typical of classical A-T, despite relative sparing of the neurologic function, suggests that cerebellar cortical volume by itself has a relatively minor role in the immediate expression of the neurodegeneration of A-T. Neurologists have long noted that the neurodegeneration of A-T is not typical of other pure cerebellar degenerations, suggesting the potential contributions of extrapyramidal, brainstem, and peripheral nerve degeneration [Crawford, 1998, 2000; Farr et al., 2002]. Furthermore, some strains of *Atm*-knockout mice show degeneration of dopaminergic neurons of the substantia nigra in the basal ganglia [Eilam et al., 1998, 2003]. Importantly,

however, *Atm*-null mice barely show any signs of cerebellar degeneration and neuromotor deficiency [Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996; Borghesani et al., 2000]. The reasons for the marked difference between the human and murine neurologic phenotypes associated with ATM deficiency are not clear.

Lack of association between homozygous protein-null *ATM* mutation leading to impaired DNA damage response, and reduced cerebellar volume on the one hand, and the severity of clinical neurologic impairment on the other hand, even in single kindred, are of potential therapeutic significance. This finding points to the presence of additional factors in the pathogenesis of functional neurodegeneration that, once found, may be important targets for therapeutic intervention.

Our observations in this family are of importance for the diagnosis of A-T. Early in the course of A-T it is frequently misidentified as cerebral palsy [Cabana et al., 1998]. The wrong diagnosis denies A-T families appropriate genetic counseling and the option of prenatal diagnosis. Mild cases are particularly problematic and require further differential diagnosis between A-T and A-TLD. Laboratory studies based on cytogenetic analysis or assessment of radiosensitivity using clonal growth are labor intensive and prone to considerable variability. On the other hand, examination of ATM and Mre11 levels using Western blotting analysis should provide the necessary information in the vast majority of cases. Rarely would a patient with classical A-T or mild A-T show normal intensity of the ATM band on a Western blot [Becker-Catania et al., 2000; Chun et al., 2003] and the same is probably true for the Mre11 band in A-TLD patients [Delia et al., 2004; Taylor et al., 2004; Fernet et al., 2005]. The only technical difficulty associated with this test is the requirement for establishing a cell line from the patient, since ATM levels in peripheral lymphocytes are very low. However, once a lymphoblastoid or fibroblast cell line is established, the analysis is technically simple and is likely to yield a definitive diagnostic result. The family described in this study provides a striking example in this regard.

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