Ataxia telangiectasia mutated–deficient B-cell chronic lymphocytic leukemia occurs in pregerminal center cells and results in defective damage response and unrepaird chromosome damage

Tatjana Stankovic, Grant S. Stewart, Christopher Fegan, Paul Biggs, James Last, Philip J. Byrd, Russell D. Keenan, Paul A. H. Moss, and Alexander M. R. Taylor

B-cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous disease involving more than one molecular mechanism that leads to the transformation of CD5⁺ B cells at either the pregerminal or postgerminal center stage of differentiation. It was previously demonstrated that ataxia telangiectasia mutated (ATM) gene mutations can occur in B-CLL and cause a defect in the p53 pathway. Here the role of ATM mutations in the pathogenesis of B-CLL is addressed. Of 50 B-CLL tumors with fully analyzed ATM and TP53, 16 had ATM mutations. Six of 50 B-CLLs showed mutations in TP53 and the remaining 28 tumors had wild-type ATM or TP53. No tumor had both ATM and TP53 mutations. Remarkably, all 16 ATM mutant B-CLLs showed the absence of somatic variable region heavy chain hypermutation indicating a pregerminal center cell origin and a common pathogenesis for these tumors. Furthermore, in 5 of the 16 B-CLLs, ATM mutation preceded the transformation stage of differentiation. At the cellular level, ATM mutant tumors exhibited a deficient ATM-dependent p53 response to gamma irradiation, failure to up-regulate TRAIL-R2, a downstream target that links irradiation-induced p53 response with apoptosis, and an inability to repair induced chromosome breaks. Mantle cell lymphoma (MCL) is also of pregerminal center origin and ATM mutations are frequent in this malignancy. It is concluded that ATM is likely to play an important role at the pregerminal center stage and a model is proposed where loss of ATM function during B-cell ontogeny drives B-CLL tumorigenesis in pregerminal B cells by a dual defect in p53 damage response and repair of chromosome breaks. (Blood. 2002;99:300-309) © 2002 by The American Society of Hematology

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in the western world. It is a heterogeneous disease with variable phenotypic and biologic features. Two distinct subsets of B-CLL have recently been defined by the stage of B-cell differentiation in which they are transformed. B-CLL can develop either in B cells that have not entered the germinal center and, consequently, do not show the presence of VH somatic hypermutation or, from postgerminal center B cells that show frequent changes within their consensus VH sequences. B-CLLs with and without VH somatic hypermutation have different clinical outcomes and it was suggested that the VH status of B-CLL tumors reflects different pathogenic mechanisms involved in the development of this malignancy. It is conceivable that these differences are caused by the involvement of different molecular events.

Patients with inherited inactivation of both alleles of the ataxia telangiectasia mutated (ATM) gene show a high predisposition for the development of leukemia and lymphoma and hypersensitivity to agents causing DNA double-strand breaks (DSBs). The ATM protein has been proposed to function in multiple biochemical pathways linking the recognition and repair of DNA DSBs to downstream cellular processes, such as cell cycle regulation and programmed cell death. Some of the biologic functions of ATM are exerted through p53 phosphorylation and activation of the p53 pathway. Although there is some overlap in the spectrum of DNA damage to which ATM and p53 respond, ATM is restricted to response to DNA DSBs and is particularly important in lymphoid development during which there is frequent processing of such breaks. There is a predominance of T-cell over B-cell tumors in A-T individuals, with chromosome translocations involving T-cell receptor (TCR) genes contributing to the development of T-cell tumors. ATM also has a role, through chromosome translocation, in the pathogenesis of sporadic T prolymphocytic leukemia (T-PLL). The mechanism of development of B-cell tumors in A-T individuals is largely unknown.

We showed recently that ATM mutations are frequent in sporadic B-CLL and that they can cause a p53 defect in response to irradiation in the absence of TP53 mutations. However, despite the observation that ATM mutations can be observed in B-CLL tumors, the relationship between ATM inactivation and pathogenesis of sporadic B-CLL is still unclear. In the present study we have addressed the possibility that inactivation of the ATM gene represents a molecular event that gives rise to a distinct subset of sporadic B-CLL tumors with a common pathogenesis. We show that ATM mutations are restricted to B-CLL with pregerminal center characteristics. We provide evidence that in these tumors, ATM inactivation occurs independently of TP53 gene
alterations and that functional loss of ATM determines a distinctive cellular response to DNA damage. Our results suggest a role for ATM in preventing both the accumulation of cells carrying DNA damage during B-cell differentiation and the consequent development of tumors at the pregerminal center stage.

Patients, materials, and methods

Patients

Peripheral blood was obtained after fully informed consent from 50 unselected patients with classical CD5+ and CD23+ B-CLL. There were 12 females and 38 males in our group of B-CLL patients. The median age of patients with ATM mutant tumors was 72.5 years (range, 39-90 years), whereas the median age in the group of patients with ATM wild-type tumors was 72.6 (range, 40-92 years). All B-CLL patients were followed for at least 2 years; the longest follow-up was 15 years. The median follow-up was 5.7 years (range, 3-10 years) for patients with ATM mutant B-CLL tumors and 7.6 years (range, 2-15 years) for patients with ATM wild-type B-CLL tumors.

Stage of disease was defined according to National Cancer Institute (NCI) criteria. Four patients with ATM mutant B-CLL were classified as in early disease (stage A) and 12 were classified as being at either stage B or C.

Among patients with ATM/TP53 wild-type tumors, 14 were classified as stage A and 14 as stage B or C. Five patients with TP53 mutant tumors were at either stage B or C at the time of analysis (Tables 1, 2, and 3). Nine of 16 patients with ATM mutant B-CLL, 11 of 28 patients with ATM/TP53 wild-type B-CLL, and 5 patients with TP53 mutant B-CLL were treated prior to collection and subsequent analysis of material (Tables 1-3).

Cell separation, DNA/RNA extraction

The mononuclear cell fraction, containing tumor cells, was separated on density gradient (Lymphoprep, Axis-Shield, PoCAs, Oslo, Norway) and frozen viable in 90% fetal calf serum (FCS)/10% dimethyl sulfoxide (DMSO). Cell samples were thawed at 37°C, washed twice in RPMI medium and cultured overnight in RPMI/10% FCS cells prior to use in damage response assays or RNA and DNA extraction.

For the separation of B and T lymphocytes from case B-CLL 33, fresh mononuclear cells were stained with either CD19PE or CD3PE antibody (Beckman-Coulter) prior to magnetic selection using antiphycoerythrin (PE) supermagnetic microbeads, and the Posseld selection program on an AutoMACS cell separator (Miltenyi-Biotec, Bergisch, Gladbach, Germany). Purity was assessed by flow cytometry using a Coulter EPICS XL. Monocytes were purified by forward and side scatter gating and collection

Table 1. Genotypic and phenotypic features of B-CLL tumors with ATM mutations

<table>
<thead>
<tr>
<th>Tumor</th>
<th>ATM protein</th>
<th>ATM mutation base (amino acid change)</th>
<th>Germline/ somatic</th>
<th>TP53 gene</th>
<th>p53/MDM2/p21 induction</th>
<th>Nearest VH/D/J family</th>
<th>% Homology</th>
<th>Karyotype</th>
<th>Stage</th>
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<tr>
<td>B-CLL1</td>
<td>A</td>
<td>1058del(GT) G5464A(E1822Q)</td>
<td>G Acq</td>
<td>WT</td>
<td>D</td>
<td>V1-02</td>
<td>100</td>
<td>45XY-13;der(21)(p13.21) (q12;p10)/46XY.add(4)(p1)/46XY</td>
<td>B C</td>
<td></td>
</tr>
<tr>
<td>B-CLL33</td>
<td>A</td>
<td>G8600GA (G2867E)</td>
<td>Acq Pre-l</td>
<td>WT</td>
<td>D</td>
<td>V3-11</td>
<td>100</td>
<td>46XX.del11q23</td>
<td>C E/C</td>
<td></td>
</tr>
<tr>
<td>B-CLL34</td>
<td>A</td>
<td>LOH across ATM 2114insA</td>
<td>Acq</td>
<td>WT</td>
<td>D</td>
<td>V3-23</td>
<td>99.6</td>
<td>46XY.del6(6;15)(p23;q14); der18.1(18.1)(q11;31)</td>
<td>C C</td>
<td></td>
</tr>
<tr>
<td>B-CLL2</td>
<td>A</td>
<td>G1048T(A350T) T1055C(J352T)</td>
<td>Acq</td>
<td>WT</td>
<td>ND</td>
<td>V1-02</td>
<td>100</td>
<td>ND</td>
<td>C/F</td>
<td></td>
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<td>R</td>
<td>G6820A (A2274K) 6277delC</td>
<td>Acq</td>
<td>WT</td>
<td>V1-69</td>
<td>D3-22</td>
<td>100</td>
<td>ND</td>
<td>A No</td>
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<td>A8266T(K2756stop)</td>
<td>G Acq</td>
<td>WT</td>
<td>D</td>
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<td>99.4</td>
<td>46XX</td>
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<td>D</td>
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<td>WT</td>
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<td>C1300F (P434S)</td>
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<td>C1004T</td>
<td>D</td>
<td>V1-69</td>
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<td>ND</td>
<td>D2-10</td>
<td>D3-10</td>
<td>V4-59</td>
<td>98.9</td>
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<td>C C</td>
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<td>3910insA</td>
<td>Acq</td>
<td>WT</td>
<td>N</td>
<td>V3-73</td>
<td>100</td>
<td>ND</td>
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<td>R</td>
<td>4829delG</td>
<td>ND</td>
<td>WT</td>
<td>N</td>
<td>V1-69</td>
<td>99.1</td>
<td>ND</td>
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<td>7511del26</td>
<td>Acq</td>
<td>WT</td>
<td>D3-10</td>
<td>V3-33</td>
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<td>3910del7</td>
<td>Acq</td>
<td>WT</td>
<td>N</td>
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<td>46XY.del13q12;3q22 or (q14;34)</td>
<td>A No</td>
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*B-CLL 38 also has sequence change A1704G (silent).
†B-CLL 38 also has sequence change C1316G (P1054R), a rare polymorphism, observed in breast tumors.
‡B-CLL 38, 39, and 40 showed a normal induction of p53, but defective induction of MDM2 and p21 following IR. WT indicates wild type; ND, not done; A, absent; R, reduced; N, normal; D, defective; Acq, acquired; G, germline; Pre-l, prelymphoid; ns, not specified; Th, previous therapy; C, chlorambucil; P, prednisolone; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisolone; F, fluorarabine; E, etoposide; and No, previous therapy given; LOH, loss of heterozygosity.
of CD14<sup>++</sup> cells. In B-CLL 34 CD19<sup>+</sup> tumor cells were separated by magnetic beads coated with CD19 antibody (Dynal).

Patients' germline DNA was extracted from buccal cavity epithelial cells. Lymphoblastoid lines from a patient and 2 healthy individuals were also used for control purposes.

Mutation analysis

Mutation analysis of the ATM coding sequence was performed on the tumor cDNA by reverse transcriptase–polymerase chain reaction (RT-PCR) and restriction endonuclease fingerprinting (REF),<sup>14,15</sup> or by complete sequencing. Most of the mutations were verified in tumor DNA. Mutation analysis of the TP53 gene was performed using either amplification and sequencing of the whole TP53 coding sequence from the tumor cDNA, or alternatively, by amplification and sequencing of exons 4 to 10 from tumor DNA using primers previously described.<sup>13</sup> The allelic status of both ATM and TP53 genes (homozygous/heterozygous/hemizygous) was determined by the presence or absence of the second allele on REF gels, sequence chromatograms, and when possible, by microsatellite analysis of the paired tumor and germline DNA.

Haplotypic analysis

Multiallelic short tandem repeats from DNA samples were analyzed for 8 loci, D11S1787, 1819, 2179, 1778, 1294, 1818, 2180, and 2178, on chromosome 11q22-23 as described before.<sup>15</sup>

Variable diversity joining region amplification and analysis of VH somatic mutations

IgH variable density joining region (VDJ) rearrangements were amplified using primers from the consensus sequences of the framework 1 (FR1) and joining region (JH) of the immunoglobulin heavy chain gene. Products were column-purified (Boehringer) and subjected to automated DNA sequencing using the ITP primer internal to JH1. The nucleotide sequence of each rearrangement was used to search the European Molecular Biology Laboratory (EMBL) and GenBank databases and the variable region database (VBASE) directory of all human germline variable region sequences.<sup>17,18</sup> Sequences that had fewer than 2% differences compared with the germline sequence of their V gene family were taken to indicate tumors lacking somatic hypermutation.<sup>17</sup>

To identify the presence of tumor cells in the nonlymphoid compartment in B-CLL 34, VDJ rearrangements were amplified using a primer corresponding to the consensus sequence of the framework 3 IgH region (FR3A) together with JH1 primer.

Protein analysis

Samples containing more than 95% viable cells, as assessed by trypan blue exclusion, were included in experiments. Cells were cultured overnight in 24-well plates with RPMI medium and 10% FCS, irradiated with 5 Gy gamma rays, kept at 37°C and harvested at defined time points.

The lysates were obtained by sonication of cells in 8 M urea, 150 mM β-mercaptoethanol, 50 mM Tris HCl, pH 7.5, and centrifugation for 30 minutes at 9°C. Samples were subjected to electrophoresis on 6% to 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels, blotted on nitrocellulose membranes, and screened for protein expression. A single nitrocellulose membrane was cut into adequate segments in order to perform simultaneous Western blotting using the ECL system (Amersham) and the following antibodies: ATM, rabbit FP8r15; p53, sheep Ab, donated by D. P. Lane (University of Dundee); MDM2, mAb 2A10, donated by R. Grand (University of Birmingham); and p21 rabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA). A separate gel was loaded for Western blotting with p53 serine 15P, rabbit Ab (Santa Cruz Biotechnology), and TRAIL-R2, goat Ab (Alexis). To verify that equivalent amounts of each sample were loaded, the filters were additionally probed with anti–actin-IgG (AC74, Sigma). Densitometry was performed routinely on each of the tested proteins as well as actin bands. A B-CLL tumor was assumed to have reduced ATM if expression of the protein was below the level of ATM expression in a group of 28 tumors with wild-type ATM/TP53.

Assessment of apoptosis

Apoptosis was measured by 2 independent methods: (1) propidium iodide (PI) incorporation and FACS analysis, and (2) staining with acridine orange and morphologic assessment. For PI staining and FACS analysis, cells were irradiated with gamma rays (5 Gy), harvested at defined time points, washed twice in phosphate-buffered saline (PBS), briefly fixed in 70% ethanol, and mixed with an equal volume of PI (10 mg/mL). Cell cycle analysis was performed using a Coulter XL flow cytometer. The apoptotic cell fraction was detected in the red fluorescent sub-G1 peak separate from cells with either G1 or S/G2/M content. Ratios of the areas of the sub-G1 and G1 peaks were obtained for each time point after irradiation and expressed as surviving fraction.

For acridine orange staining, cells were mixed with an equal volume of acridine orange (10 mg/mL) and at each time point the proportion of cells showing fragmentation of nuclei was determined by microscopy.

Chromosome radiosensitivity and karyotype analysis

Peripheral blood from patients with B-CLL was cultured for 5, 6, or 7 days in RPMI medium containing 10% FCS, glutamine, penicillin, streptomycin, and 0.1 mg/mL phorbol myristate acetate (PMA) (Sigma). Cells were irradiated with 1 gray x-rays (1 Gy per minute) and harvested 4 hours later. Chromosome preparations were made, stained with Giemsa, and the G2 x-ray–induced damage was analyzed. Chromosome analysis of B-CLLs, for karyotyping, was carried out using standard G-banding techniques following culturing of the B-CLL tumor cells in PMA for 5, 6, or 7 days.
Results

**ATM mutations are independent of TP53 mutations in B-CLL**

In a group of 50 B-CLL tumors (different from that in Pettitt et al\(^1\)), where sufficient cells were available for a range of assays, we analyzed the status of TP53 and ATM using the REF method in combination with the sequencing of the entire ATM coding region. ATM mutations were identified in 16 B-CLLs, none of which also had a TP53 mutation (Table 1). In 2 of these tumors (B-CLL 33 and B-CLL 36) there was evidence for the loss of one ATM allele and the presence of mutation in the other. In an additional 5 tumors (B-CLLs 1, 34, 2, 4, and 7), 2 ATM mutations were identified. Five tumors (B-CLLs 35, 37, 38, 39, and 40) each had a single point mutation, 2 of which (in B-CLL 35 and B-CLL 40) were predicted to lead to the premature termination of the protein. The missense mutations identified were in regions conserved between mouse and man and not previously reported as polymorphisms. Finally, 4 tumors were detected carrying a single small deletion or insertion predicted also to lead to protein truncation (B-CLLs 6, 41, 42, and 5).
A separate group of 6 B-CLLs (B-CLLs 9, 43, 44, 45, 46, and 66), each with a single TP53 mutation, was identified. Three tumors were hemizygous and the other 3 heterozygous for a TP53 mutation. The patient with B-CLL 46 had previously been diagnosed as having Li-Fraumeni syndrome and, as expected, the TP53 mutation was present in the germline (Table 2). The remaining 28 B-CLL tumors showed absence of both ATM and TP53 mutations (Table 3).

Compared with the ATM/TP53 wild-type B-CLLs, the ATM mutant subset showed a higher percentage of stage B and C tumors (75% vs 50%). This difference, however, was not statistically significant (P = .125, Fisher exact test).

Although based on relatively small numbers, our results raise the possibility that the simultaneous occurrence of ATM and TP53 mutations is either a mutually exclusive event, or certainly rare, in the development of B-CLL.

**ATM mutant tumors are of pregerminal center B-cell origin lacking VH somatic hypermutation**

In order to determine whether all ATM-mutated B-CLLs were derived from the same stage of B-cell differentiation, we analyzed the VH status of the ATM mutant tumors and compared this with ATM/TP53 wild-type tumors.

Clonal IgH VDJ rearrangements were amplified and sequenced from 50 B-CLL tumors previously characterized for the presence of either the ATM or TP53 mutation. Sequencing revealed that all 16 tumors with ATM inactivation showed absence of somatic hypermutation (Table 1) suggesting that these were derived from a progenitor bearing characteristics of pregerminal center B cells.

Absence of VH somatic mutations was also noted in all 5 tumors with acquired TP53 mutations, whereas the sixth TP53 mutant B-CLL from a patient with Li-Fraumeni syndrome showed the presence of VH somatic hypermutations (Table 2). In contrast, the majority of tumors with wild-type TP53/ATM (20 of 28) showed the presence of multiple somatic mutations in their VH regions (Table 3).

Our finding is consistent with a common mechanism of tumorigenesis in B-CLL tumors with ATM inactivation.

**ATM mutations can be acquired early during tumorigenesis in B-CLL**

If ATM inactivation is involved in B-CLL transformation at the pregerminal B-cell stage, mutations should be acquired prior to this point. We tested this possibility by searching for ATM mutations at 2 points: in the patients’ germlines and in non-B cells.

First, ATM germline mutations were identified in 3 patients with B-CLL (B-CLLs 1, 4, and 35). The previously reported mutation 1058delGT in B-CLL 1 and mutation A8266T detected in B-CLL 35, were truncating and could not be polymorphisms. In addition, we identified mutation A8266AT in an A-T family (Table 1). Microsatellite marker analysis (data not shown) provided convincing evidence that B-CLL 35 carried the same haplotype in the region of the ATM gene as the affected individual from family 63, giving provenance to the pathogenic nature of this mutation. G6820A observed in the germline of B-CLL 4 was likely to be a real mutation as we were not able to identify this change in DNA from 140 normal meioses.

Second, we were able to obtain separated tumor and nontumor mononuclear cells in 2 additional patients with B-CLL (B-CLLs 33 and 34). Truncating mutation 2114insA in B-CLL 34 was present in both tumor cells and the nontumor mononuclear compartment containing T cells, NK cells, and monocytes, but not in the buccal smear DNA (Figure 1). A clonal IgH rearrangement could be detected in the tumor population, but not in nontumor cells (Figure 1), suggesting that cross contamination of the nontumor cells with tumor cells was unlikely to be the cause of the presence of the ATM mutations in this compartment. Rather, the results suggested that the mutation 2114insA arose in a progenitor at a stage prior to B-lymphoid commitment.

B-CLL 33 carried the G8600A mutation, which is predicted to cause a significant amino-acid change in the ATM PI-3 kinase domain. This case was used to define the hematopoietic progenitor targeted by this mutation. The following fractions of high purity were obtained: CD19+ separated cells containing mostly B-CLL tumor cells; CD14+ sorted cells containing monocytes, and CD3+ sorted cells containing T lymphocytes (Figure 2). Both the tumor B cells and the monocyte fraction showed the presence of the G8600A mutant allele alone, whereas the pure T cells, in addition to the mutant allele, revealed a small peak of the normal sequence (Figure 2). The prominence of the mutant allele in the DNA sequences of all analyzed
ATM mutations and protein expression. Four tumors (B-CLLs 1, 2, 33, and 34) with apparent alterations of both ATM alleles showed a complete absence of ATM expression (Table 1). In the remaining 12 tumors with ATM mutations, protein expression was variable: 8 tumors showed a clearly reduced expression of ATM compared with the level of ATM protein expression in 28 ATM wild-type tumors, and 4 B-CLLs showed normal ATM expression. It is noteworthy that in each of the 4 tumors with normal ATM expression a single missense mutation was identified. In one of these, B-CLL 36, the mutant ATM allele was the only allele present as there was an 11q deletion and LOH across the ATM gene region, presumably reflecting loss of the normal allele.

ATM mutations and p53 cellular response to IR. Our previous study with a different group of B-CLL tumors demonstrated that ATM mutations can cause a defect in p53 response to DNA damage induced by ionizing radiation.13 We used this observation to analyze the pathogenicity of ATM mutations in the present study. Of 15 ATM mutant tumors that could be tested, 8 showed a clear defect in both p53 accumulation and its transcriptional activity as measured by p21/MDM2 up-regulation. This included 2 tumors with 11q deletion and mutation in the other ATM allele (B-CLLs 33 and 36).

Interestingly, 3 ATM mutant tumors showed defective p21/MDM2 up-regulation despite the high accumulation of p53 (Table 1, Figure 3). In addition, 4 B-CLL tumors, each with a single truncating ATM mutation, exhibited high p53/p21/MDM2 up-regulation (data not shown). As expected, p53/p21/MDM2 up-regulation was also high in 19 analyzed B-CLL tumors with ATM/TP53 wild type (Table 3, Figure 3).

We conclude that ATM mutations in the majority of B-CLL tumors (11/15), led to a defective p53 response to IR-induced DNA damage, regardless of the level of ATM expression.

Resistance to IR-induced apoptosis in ATM-deficient B-CLL tumors is associated with defect in TRAIL-R2 up-regulation. All 4 ATM mutant tumors completely lacking ATM expression in this series showed severely reduced apoptosis following IR (Figure 4A). Furthermore, in 2 representatives of these tumors, despite some IR-induced p53 accumulation, there was a clear absence of p53 serine-15 phosphorylation (Figure 4B) at the time point used, consistent with the well-documented delay in p53 serine-15 phosphorylation in classical A-T cells. This modification is dependent on ATM and crucial for apoptotic activity of p53.19

We investigated the link between an aberrant IR-induced p53 response and decreased IR-induced apoptosis in B-CLL tumors. Many downstream targets have been proposed to mediate the apoptotic functions of the p53 protein, including the death effector Bax. The lack of ionizing radiation-induced up-regulation of Bax in any of our group of B-CLL tumors tested (data not shown) prompted us to investigate whether a defective induction of other p53-dependent downstream proapoptotic targets could account for the apparent resistance to ionizing radiation-induced apoptosis in B-CLLs with ATM gene abnormalities.

Recently, transcriptional up-regulation of TRAIL Receptor 2 (TRAIL-R2) was reported to be a p53-dependent response specifically restricted to cells undergoing apoptosis and was shown to be

Figure 2. Prelymphoid origin of G8600A mutation in B-CLL 33. (A) G8600A mutation in DNA from tumor cells of patient with B-CLL 33 (i), separated to 99.8% purity using CD19 PE-coated magnetic beads (ii). (B) G8600A mutation together with a small peak of a normal sequence in DNA from CD3+ cells of patient with B-CLL 33 (i), separated to 95% purity using CD3 PE-coated magnetic beads (ii). (C) G8600A mutation in DNA from CD14+ cells of patient with B-CLL 33 (i) separated to 100% purity using CD14 labeling and FACS sorting (ii). (D) Absence of G8600A mutation in patient’s buccal epithelial cells.

subpopulations and the absence of the mutation in the germline from the buccal smear, suggested that all 3 blood lineages (T, B, and monocyte) were derived from a single hematopoietic precursor carrying the G8600A mutation together with the loss of a normal ATM allele. The presence of a minor germline peak in the T-cell population is consistent with a subpopulation of long-living T cells existing before the evolution of a lineage noncommitted progenitor carrying the G8600A mutation. We concluded that in B-CLL 33 not only ATM mutation, but complete ATM inactivation, was acquired early during hematopoiesis, well before the stage of tumor development, as a result of affecting more than one lineage.

Cellular features of ATM mutant B-CLLs

We first asked whether all ATM mutations in the B-CLLs were pathogenic. We took as possible indicators of a pathogenic effect (1) a decrease in ATM protein expression, and (2) a functional assay showing defective p53 response following exposure of the tumor cells to ionizing radiation (IR). In tumors with pathogenic ATM mutations, we then focused on 2 aspects of the cellular phenotype: identification of a common defect in the downstream target which provides a link between aberrant IR induction of p53 and resistance to apoptosis in these cells and the ability of ATM mutant tumor cells to repair chromosome damage following gamma irradiation.

subpopulations and the absence of the mutation in the germline from the buccal smear, suggested that all 3 blood lineages (T, B, and monocyte) were derived from a single hematopoietic precursor carrying the G8600A mutation together with the loss of a normal ATM allele. The presence of a minor germline peak in the T-cell population is consistent with a subpopulation of long-living T cells existing before the evolution of a lineage noncommitted progenitor carrying the G8600A mutation. We concluded that in B-CLL 33 not only ATM mutation, but complete ATM inactivation, was acquired early during hematopoiesis, well before the stage of tumor development, as a result of affecting more than one lineage.

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ATM mutations and protein expression. Four tumors (B-CLLs 1, 2, 33, and 34) with apparent alterations of both ATM alleles showed a complete absence of ATM expression (Table 1). In the remaining 12 tumors with ATM mutations, protein expression was variable: 8 tumors showed a clearly reduced expression of ATM compared with the level of ATM protein expression in 28 ATM wild-type tumors, and 4 B-CLLs showed normal ATM expression. It is noteworthy that in each of the 4 tumors with normal ATM expression a single missense mutation was identified. In one of these, B-CLL 36, the mutant ATM allele was the only allele present as there was an 11q deletion and LOH across the ATM gene region, presumably reflecting loss of the normal allele.

ATM mutations and p53 cellular response to IR. Our previous study with a different group of B-CLL tumors demonstrated that ATM mutations can cause a defect in p53 response to DNA damage induced by ionizing radiation.13 We used this observation to analyze the pathogenicity of ATM mutations in the present study. Of 15 ATM mutant tumors that could be tested, 8 showed a clear defect in both p53 accumulation and its transcriptional activity as measured by p21/MDM2 up-regulation. This included 2 tumors with 11q deletion and mutation in the other ATM allele (B-CLLs 33 and 36).

Interestingly, 3 ATM mutant tumors showed defective p21/MDM2 up-regulation despite the high accumulation of p53 (Table 1, Figure 3). In addition, 4 B-CLL tumors, each with a single truncating ATM mutation, exhibited high p53/p21/MDM2 up-regulation (data not shown). As expected, p53/p21/MDM2 up-regulation was also high in 19 analyzed B-CLL tumors with ATM/TP53 wild type (Table 3, Figure 3).

We conclude that ATM mutations in the majority of B-CLL tumors (11/15), led to a defective p53 response to IR-induced DNA damage, regardless of the level of ATM expression.

Resistance to IR-induced apoptosis in ATM-deficient B-CLL tumors is associated with defect in TRAIL-R2 up-regulation. All 4 ATM mutant tumors completely lacking ATM expression in this series showed severely reduced apoptosis following IR (Figure 4A). Furthermore, in 2 representatives of these tumors, despite some IR-induced p53 accumulation, there was a clear absence of p53 serine-15 phosphorylation (Figure 4B) at the time point used, consistent with the well-documented delay in p53 serine-15 phosphorylation in classical A-T cells. This modification is dependent on ATM and crucial for apoptotic activity of p53.19

We investigated the link between an aberrant IR-induced p53 response and decreased IR-induced apoptosis in B-CLL tumors. Many downstream targets have been proposed to mediate the apoptotic functions of the p53 protein, including the death effector Bax. The lack of ionizing radiation-induced up-regulation of Bax in any of our group of B-CLL tumors tested (data not shown) prompted us to investigate whether a defective induction of other p53-dependent downstream proapoptotic targets could account for the apparent resistance to ionizing radiation-induced apoptosis in B-CLLs with ATM gene abnormalities.

Recently, transcriptional up-regulation of TRAIL Receptor 2 (TRAIL-R2) was reported to be a p53-dependent response specifically restricted to cells undergoing apoptosis and was shown to be
defective in LCLs derived from classical A-T patients following exposure to IR. We determined first whether TRAIL-R2 was up-regulated in B-CLL cells without ATM mutations and then whether this was lost in ATM mutant B-CLLs. Western blot analysis revealed a peak of TRAIL-R2 up-regulation in an ATM/TP53 wild-type B-CLL 8 hours to 10 hours following gamma irradiation (Figure 4C).

We next investigated whether ATM mutant tumors either with a complete p53/p21/MDM2 defect or with a defect in p21/MDM2 up-regulation, but with p53 accumulation, had the same impact on TRAIL-R2 up-regulation. Twenty B-CLL tumors from which material was available were analyzed at 0 hours and 10 hours after irradiation (Figure 3). Five B-CLLs (B-CLLs 1, 7, 33, 34, and 35) with a complete defect in p53 response, 2 B-CLLs (B-CLLs 38 and 39) with normal p53 response but defective p21/MDM2 up-regulation, and one TP53 mutant (B-CLL 9), clearly failed to up-regulate TRAIL-R2 10 hours after irradiation (Figure 3). In comparison, the 12 B-CLL tumors with ATM/TP53 wild type (B-CLLs 13, 19, 20, 50, 52-55, 57, 59-61) showed strong up-regulation of TRAIL-R2. Five cases representative of the 12 analyzed ATM/TP53 wild-type tumors (B-CLLs 20, 52, 13, 19, and 50) are shown in Figure 3.

We conclude that TRAIL-R2 is a p53 downstream target linking IR-induced p53 response to apoptosis, and that this response is defective in both ATM and TP53 mutant B-CLL tumors.

ATM-deficient B-CLL tumors show reduced ability to repair chromosome damage. Typical B-CLL is not characterized by the presence of translocations involving the immune system genes. Indeed, none of the 9 ATM mutant tumors for which karyotype was available showed the presence of translocations involving the

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Figure 3. Ionizing radiation-induced accumulation of p53, p21, MDM2, and TRAIL-R2 at 0 hours and 10 hours following IR in ATM mutant B-CLLs. B-CLLs 34, 33, 7, 1, and 35 are representative of 8 tumors with ATM mutations showing defective p53, p21, and MDM2 responses following exposure to IR. B-CLLs 38 and 39 are ATM mutant tumors with normal p53 accumulation but defective p21 and MDM2 up-regulation. A B-CLL tumor (B-CLL 9) with TP53 mutation showed an elevated basal p53 protein level but a defect in the ability to accumulate p53 and up-regulate p21 and MDM2. B-CLLs 20, 52, 19, 50, and 13 are representative of 19 analyzed tumors with wild-type ATM and p53 which showed normal p53, p21, and MDM2 responses. The TRAIL-R2 response 10 hours after irradiation was defective in ATM-deficient tumors (B-CLLs 33, 34, 38, 39, 7, and 35) as well as in TP53 mutant (Mut) B-CLL 9, but it was normal in B-CLLs 20, 52, 19, 50, and 13 representative of 12 B-CLL tumors with ATM/TP53 wild type (WT) analyzed for TRAIL-R2 response. The actin expression shows equal loading.

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Figure 4. Defect in cellular responses in ATM mutant B-CLLs linking IR-induced p53 defect with resistance to apoptosis. (A) The rate of apoptosis after exposure to 5 Gy gamma irradiation was compared in B-CLL tumors either with ATM mutations, TP53 mutations, or with wild-type TP53/ATM sequence. Mean levels of apoptosis induced by ionizing radiation in B-CLLs with wild-type ATM (B-CLLs 13, 19, and 54), mutant ATM (B-CLLs 1, 2, 33, and 34), and also mutant p53 (B-CLLs 9 and 43) up to 6 days after irradiation. In unirradiated B-CLL cells the level of apoptosis was indistinguishable between the different genotypes at all 4 time points (data not shown). (B) Phosphorylation at serine-15 of p53 in B-CLLs 20, 13, and 19 with wild-type ATM but not in B-CLLs 33 and 34 with mutant ATM, or in TP53 mutant B-CLL 9 at 0 hours and 10 hours following irradiation. (C) Expression of TRAIL-R2 at 0, 2, 4, 6, 8, and 10 hours following IR with 5 Gy. Up-regulation reaches a peak 10 hours following irradiation in B-CLL20 with wild-type ATM. ATM mutant B-CLL 1 failed to up-regulate TRAIL-R2 following irradiation.
immmunoglobulin genes (Table 1), suggesting that errors during VDJ recombination did not play a role in their pathogenesis.

We asked, therefore, how effective tumor cells were in the repair of general non site-specific DNA damage induced by IR. B-CLLs either with total loss of ATM and defective induction of p53, p21/MDM2/TRAIL-R2 (B-CLLs 1, 33, and 39) or mutant ATM with defective activation of p21/MDM2 (B-CLL 39) were irradiated. Of these irradiated ATM mutated B-CLL tumor cells, 74% showed unrepaird chromosome damage compared with 23% cells from 2 B-CLLs (51 and 56) with ATM/TP53 wild-type and normal p53 responses (Figure 5). In comparison, 16% of phorbol myristate acetate (PMA)–stimulated peripheral blood lymphocytes from 4 healthy individuals showed chromatid damage following exposure to the same dose of IR (not shown). ATM mutant B-CLLs, therefore, in addition to a defective p53 response to IR, have a defect in repair of some forms of chromosome damage.

**Discussion**

In this study we have shown that inactivation of the ATM gene results in a combination of characteristics that defines a distinct subset of B-CLL tumors. The restriction to the same stage of differentiation, the timing of ATM mutations and their occurrence independently of TP53 mutations, all suggest that an ATM defect plays a role in the pathogenesis of B-CLL. Our findings, however, do not exclude the possibility of other pathways initiating the tumor process in B-CLL as a part of the heterogeneity of this malignancy.

The most consistent feature of ATM mutant tumors in our study was the absence of VH somatic hypermutation. VDJ sequences of all 16 ATM mutant tumors showed more than 98% sequence homology compared with the appropriate VH germline (9 of these tumors showed 100% homology with the germline sequence of the corresponding VH family). Apart from the V1-69 family, previously associated with VH unmutated B-CLL tumors and detected here in 3 ATM mutant tumors, we have not observed bias toward any particular family of V genes. Two subsets of CLL that transform at distinct stages of differentiation, one with hypermutated IgH VDJ recombinations and another with unmutated recombinations, show distinct biologic behaviors with tumors derived from the nonmutated being more aggressive.1,3 ATM mutant B-CLLs revealed a tendency, although not significant in this study, to be associated with a later disease stage: 75% of tumors were at either stage B or C. It remains possible, therefore, that ATM deficiency accounts for a subset of VH unmutated tumors with an aggressive biologic behavior. The aggressive behavior of ATM-deficient B-CLLs was suggested in a previous report.21

The purpose of this study was to identify the common cellular features of ATM mutant B-CLL tumors. In order to attain this goal we used simultaneous analysis of mutations by RT-PCR, ATM protein expression, and cellular DNA damage response. All patients involved in this study had high lymphocyte counts, caused by the predominance of tumor cells. Consistent with this was the single clonal VDJH rearrangement readily detectable in peripheral blood mononuclear cells from all B-CLLs, apart from B-CLL 11.

The classic concept of tumor suppressor gene inactivation with mutation at one allele and loss of the wild-type allele does not appear to apply to the majority of ATM mutant B-CLLs. LOH across the ATM gene region associated with 11q deletion was detected in only 2 of 16 B-CLL tumors with ATM mutations. The remaining ATM mutant tumors revealed a spectrum of allelic combinations. In addition, in some tumors only a single missense or truncating mutation was detected.

It is possible that the number of tumors with inactivation of one or both ATM alleles is higher than we were able to demonstrate, since only about 70% of all ATM mutations are readily detectable12,22 irrespective of the method of detection. Experience shows that, overall, the missed mutations are likely to be missense, rather than nonsense. Although nonsense mutations can cause instability of RNA through the mechanism of nonsense-mediated mRNA decay (NMD), there is no evidence for such a mechanism operating in the ATM gene23 and this is unlikely to contribute to missed mutations. There was also no evidence that ATM-dependent cellular functions in 19 tested tumors with ATM/TP53 wild type were anything other than normal. Thus, even if present, any ATM sequence changes in these tumors were unlikely to be pathogenic mutations.

We measured the functional consequences of ATM mutations on ATM expression and the ability of ATM mutant B-CLL tumor cells to stabilize p53 and transactivate p53 downstream genes following gamma irradiation. Twelve tumors with ATM mutations showed either absent or significantly reduced ATM expression. Four tumors, B-CLLs 36, 37, 38, and 39, however, showed normal ATM protein levels despite the presence of ATM mutations. We considered various explanations for this observation. In these 4 tumors p53 up-regulation was clearly defective, indicating that the ATM sequence changes were pathogenic rather than polymorphic. ATM polymorphisms are not associated with a defect in ATM-dependent cellular responses. In all 4 tumors VDJH amplification revealed a single clonal rearrangement and in B-CLL 36, with a single missense mutation in one ATM allele and loss of the second ATM allele, there was no evidence of a normal sequence on the sequence chromatograms. We concluded that a substantial presence of a nontumor population was an unlikely cause of normal ATM expression in these tumors. Our observations favored the possibility that some mutant ATM proteins can be expressed at a high level in B-CLLs, as we previously noted in A-T individuals.15,24 In contrast, 4 tumors, with reduced level of ATM expression and each with a single identified truncating mutation, showed an apparently normal p53 damage response. The simplest explanation is that there was incomplete ATM inactivation in these tumors.

Unlike ATM and TP53 mutant tumors, all 19 analyzed tumors without apparent ATM/TP53 mutations showed normal p53 response to irradiation. Despite this, we considered the possibility that these tumors possessed some downstream defects in the p53/MDM2/p21 pathway. We examined TRAIL–R2 up-regulation as a link between IR-induced p53 response and apoptosis. All 12
analyzed ATM/TP53 wild-type tumors showed normal up-regulation of TRAIL-R2, suggesting normal induction of the IR-induced apoptotic pathway. Furthermore, in a separate study we demonstrated that 30 B-cell tumors with normal IR-induced p53 response had comparable levels of IR-induced apoptosis. Therefore, if downstream defects in the p53 pathway exist in these B-CLLs, their overall contribution to the defect in IR-induced apoptosis is likely to be small.

It is difficult to determine the precise timing of ATM mutations detected only in B-CLL tumor cells. A targeted progenitor can be more precisely defined if a mutation is present not only in a B-CLL tumor but also in the nontumor hematopoietic cells. We showed that in the patient with B-CLL 33, mutation G8600A was present in tumor cells and T cells as well as in monocytes. In both tumor cells and monocytes a complete absence of the normal sequence at the coding position 8600 suggested a loss of the second ATM allele. Therefore, in this patient, complete ATM inactivation occurred in a progenitor at the stage prior to lymphoid commitment. Mutation 2114insA was present in both CD19+ and CD19+ mononuclear cells of the patient with B-CLL 34, suggesting that this mutation probably also occurred in a cell at a stage prior to lymphoid commitment.

In each of 3 B-CLL patients (B-CLLs 1, 4, and 35), we identified a single ATM germline mutation. B-CLL is currently the only sporadic lymphoid tumor in which germline ATM mutations have been observed. If carrying ATM mutations can confer an increased risk of developing B-CLL with low penetrance, in much the same way that an increased risk of breast cancer in female A-T carriers has been suggested, it is unlikely that families with ATM mutation carriers will show multiple cases of B-CLL. Indeed, a slightly increased number of patients with CLL among relatives of mutation carriers has been suggested, it is unlikely that families with ATM mutation carriers will show multiple cases of B-CLL. Moreover, lymphoid malignancies are not prominent among tumor types that can give rise to lymphoid tumors sporadically as well as in A-T patients. It is interesting that, although the same tumor types can be observed in both A-T and non-A-T individuals (eg, T-PLL), the predominant cell of origin for tumors is different in each group. In the case of inherited ATM inactivation of both alleles, in A-T and in the ATM±/– mouse model, T-cell tumors are the most frequent (Figure 6A) and translocations involving TCR

**Figure 6. Model for development of lymphoid malignancies on the background of inherited or acquired ATM inactivation**. (A) A-T patients with inherited inactivation of both ATM alleles develop both B (B-cell lymphoma) and T-cell tumors, but T-cell tumors are largely predominant. ATM−/– mice develop only T-cell tumors. (B) Individuals with both germline ATM alleles wild type, can acquire complete ATM inactivation during lymphoid development and develop either B-cell malignancies of pregerminal center origin (B-CLL and MCL) or T-cell tumors (T-PLL). * Patients with sporadic B-CLL can be ATM mutation carriers. In contrast to A-T patients with inherited inactivation of both ATM alleles, B-cell tumors are more common than T-cell malignancies in non-A-T individuals.
genes play an important role in their development. In contrast, when acquired in hematopoietic tissue, ATM inactivation leads predominantly to the development of B-cell tumors (Figure 6B), where translocations involving IgH genes may or may not play a role. This may be related to the different role of ATM during B-cell and T-cell development. It is possible that the role of ATM in T-cell development is particularly important in the process of VDJ recombination. Although ATM inactivation does not cause a gross defect in VDJ recombination in T cells, it does not result in TCR interlocus translocations. The absence of ATM in T cells could cause tumorigenic VDJ errors. In contrast, during B-cell development, the role of ATM may not be restricted to the stage of VDJ recombination but rather is evenly distributed throughout B-cell ontogeny. This would allow ATM inactivation to initiate tumorigenesis at any point during early B-cell development. In contrast to lymphoid malignancies initiated by VDJ errors, the pathogenesis of B-CLL may involve more indolent events requiring a longer period of time to promote complete malignant transformation. ATM inactivation that drives B-CLL tumorigenesis independently of VDJ translocations would certainly fit with this concept.

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References

Ataxia telangiectasia mutated–deficient B-cell chronic lymphocytic leukemia occurs in pregerminal center cells and results in defective damage response and unrepaired chromosome damage

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