Deficiency of the ATM Protein Expression Defines an Aggressive Subgroup of B-Cell Chronic Lymphocytic Leukemia

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Abstract

The gene mutated in ataxia telangiectasia, ATM, on human chromosome 11q22–q23, is implicated in cell cycle control and DNA repair. Ataxia telangiectasia patients as well as ATM-deficient mice are immune deficient and develop lymphoproliferative disease. Abnormalities in 11q22–q23 have also been described in B-cell chronic lymphocytic leukemia (B-CLL). We analyzed B-CLL samples for loss of heterozygosity (LOH) using microsatellite markers located at the ATM (D11S2179), mixed-lineage leukemia (MLL; D11S1356), and BCL1 (D11S987) loci, all of which are located around 11q23. Five (14%) of 36 informative cases showed LOH at the ATM gene, and two of these five cases had LOH at the MLL gene. No LOH was detected at the BCL1 locus, and none of the cases showed LOH at the MLL gene without LOH at the ATM gene. Four of these five cases with LOH at the ATM gene were studied for expression of ATM protein by Western blot analysis and RIA. Thirty-eight (34%) of these cases showed ATM levels <50% of that seen in normal lymphoid cells. No morphological or immunophenotypic difference was observed between ATM-deficient B-CLL cases and cases with normal ATM expression. However, patients with ATM deficiency had significantly shorter survival times (35.66 versus 97.3 months; $P = 0.003$) and more aggressive disease, suggesting that ATM is involved in the leukemogenesis of B-CLL. These data also suggest that the ATM gene may play a role in the reported 11q23 abnormality in B-CLL, which also characterizes an aggressive disease.

Introduction

Although B-CLL is the most common adult leukemia, no specific gene has been implicated to play a major role in this disease thus far (1–3). 53 deletion has been reported to be associated with poor prognosis in B-CLL (4). Trisomy 12 has been reported in 15–35% of cases of B-CLL (5, 6), and recent papers suggested abnormalities in genes located at 13q12 and 13q14 in ~30–80% of patients with B-CLL (7–12). Pegan et al. (13) demonstrated karyotypic abnormalities in 6% of B-CLL patients and reported association of 11q deletion with aggressive disease (13). Recent studies by Stilgenbauer et al. (14) and Döhner et al. (15) demonstrated LOH on 11q22.3–q23.1 in ~20% of B-CLL patients. Using fluorescence in situ hybridization analysis, the authors demonstrated that 11q deletions were more common than trisomy 12 in the 214 patients analyzed, and that patients with 11q deletion who were younger than 55 years had more aggressive disease and shorter survival times. These studies localized the deletion on 11q to the 2–3 Mb region that includes ATM, RDX, and FDX1 genes (14).

The ATM gene, which is responsible for the human autosomal recessive disease AT, is located on chromosome 11q23 (16). The ATM gene is composed of 66 exons spanning more than 150 kb of DNA, has the mRNA of ~13 kb, and appears to be expressed in a wide variety of cells (17–19). The gene encodes a 3056-amino acid protein with a molecular weight of 370,000 that is primarily, but not exclusively, located in the nucleus of normal human cells (18, 19). The ATM protein is critical for both cellular response to ionizing radiation and normal cell cycle progression (20). It serves as a sensitive checkpoint to control DNA damage. ATM−/− cells are hypersensitive to irradiation with X-rays and are defective in cell cycle arrest after irradiation (21). This correlates with defective up-regulation of p53, which is believed to be induced by ATM (20, 22). The cardinal feature in patients with AT is a predisposition to cancer. These patients develop cancer at a rate that is 100-fold greater than the general population (23–25). Lymphoma and acute lymphocytic leukemia constitute 85% of these cancers during childhood and 50% after age 20. The incidence of epithelial malignancies increases with age as well. Lymphoreticular malignancies develop in 10–15% of patients who are AT homozygous.

Epidemiological studies have suggested that ~1% of the general population is AT heterozygous, and these people have a certain degree of cancer predisposition, particularly to breast cancer and lymphoproliferative disease (24–27). However, recent studies have raised doubts regarding ATM involvement in breast cancer (28, 29). Two groups have failed to find increased incidence of ATM mutations in patients with breast cancer (28, 29). A recent study by Vorechovsky et al. (30) demonstrated a high frequency of ATM mutations in T-cell prolymphocytic leukemia. Unlike mutations reported in AT patients, most T-cell prolymphocytic leukemia mutations were missense mutations rather than truncating mutations (30).

We report that the ATM protein is abnormally expressed in B-CLL. We first screened a group of 41 B-CLL patients for LOH on chromosome 11q using microsatellite markers encompassing the ATM gene. After determining that the ATM gene was frequently deleted in this group of patients, we studied ATM protein expression using Western blot analysis and RIA in a group of 111 B-CLL patients. We demonstrated that the complete absence or a low expression of ATM protein identifies a subgroup of B-CLL patients with shorter survival times. Our data suggest that the ATM gene is involved in the leukemogenesis of B-CLL.

Materials and Methods

Patients and Tissue Samples. Normal and tumor tissue samples were used for microsatellite analysis, and tumor samples only from 111 patients were used for protein studies. Morphological diagnosis was performed in the Department of Laboratory Medicine and confirmed by flow cytometry and molecular studies (immunoglobulin heavy and light chain T-cell receptor and...
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BCL1 rearrangement). Protein and DNA from bone marrow or peripheral blood samples were obtained at the time of presentation if >70% of cells were CD5- and CD19-positive. Control genomic DNA was derived from bone marrow samples, taken during the period of clinical remission, that were negative for CLL cells by morphological, flow cytometric (CD5+, CD19+, <5%), and molecular studies (no immunoglobulin heavy or light chain rearrangement).

DNA Isolation. DNA was isolated from the peripheral blood or bone marrow samples, after the red cells were lysed, using GENEPURE 341 DNA extraction kit according to procedures recommended by the manufacturer (model 341; ABI, Foster City, CA). Genomic DNA was quantified by a Du-7000 (Beckman Instruments, Inc., Fullerton, CA) at an absorbance of 260 nm.

PCR Amplification of Microsatellite Repeats. The microsatellite markers D11S987 (for the cyclin D1 gene), D11S2179 (for the ATM gene), and D11S1356 (for the MLL gene; primer sequences retrieved from the Genome Database) were selected to cover regions of interest on chromosome 11. One oligonucleotide of each primer pair was labeled with either FAM, HEX, or TAMRA fluorescent dye (Perkin-Elmer, Corp., Norwalk, CT). Paired normal and tumor DNA samples from each patient were amplified simultaneously (PCR conditions as specified by Genome Database). Briefly, samples were first activated at 95°C for 12 min, then amplified at 94°C for 30 s and 60°C for 30 s; for 30 cycles. Thirty cycles were carried out on a 9600 Perkin-Elmer thermal cycler in a total volume of 50 µl. D11S987 and D11S1356 were amplified in a multiplex reaction; D11S2179 was amplified alone. PCR was performed using AmpliTaq Gold DNA polymerase.

Electrophoresis and Data Analysis. Aliquots (0.6 µl) of the PCR reaction for each microsatellite was mixed with 0.2 µl of size standard (GENESCAN 2500-ROX) and 2.5 µl of blue formamide loading buffer (ABI). Samples were denatured at 95°C for 5 min, loaded onto 6% denaturing polyacrylamide gel, and electrophoresed on a model 373 Sequencer (ABI) for 12 h. Approximately 80% of samples were also reanalyzed using ABI 310. The automatically collected data were analyzed using GENESCAN software (version 1.2) as described in the manufacturer's manual.

Protein Isolation. Mononuclear cells from peripheral blood and bone marrow samples were homogenized in ice-cold buffer (20 mM Tris/HCl (pH 7.2), 1 mM EDTA, 1 mM EGTA, 0.1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.1% leupeptin, 0.1% pepstatin, and 0.1% Triton X-100). After solubilization on ice for 1 h, the lystate was centrifuged at 35,000 rpm for 45 min, and the supernatant was collected. The protein concentration was estimated by Bio-Rad standard assay (Bio-Rad Laboratories, Richmond, CA), and 200 µg of each extract was run on 7.5% SDS-PAGE gel and stained with Coomassie blue R-250 to check the protein profile.

Immunoblot Analysis of ATM Protein. Three hundred µg of cell extract from various B-CLL patients and normal volunteers were electrophoretically separated on a 5.5% SDS-PAGE gel. The protein was then transferred to nitrocellulose paper. The nitrocellulose membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 and 0.01% sodium azide for 1 h at room temperature. The blot was then incubated overnight at 4°C with rabbit anti-ATM polyclonal antibody (Oncogene Research, Cambridge, MA), 2 µg/ml in PBS containing 2.5% nonfat milk, 2.5% BSA, and 0.1% Tween 20. The membrane was washed with PBS containing 0.1% Tween 20. The blot was then incubated with 1:2000 diluted anti-rabbit immunoglobulin linked to horseshadish peroxidase (Amersham, Arlington Heights, IL) in PBS containing 1% nonfat milk and 0.1% Tween 20. Immunoreactive bands were developed using the ECL detection system (Amersham). After ECL detection, the membrane was stripped from the primary and secondary antibodies under conditions recommended by Amersham, Inc. and then blocked and probed with an anti-β-tubulin (Ab-1) monoclonal antibody Cy3 (Oncogene Science, Inc., Uniondale, NY) or anti-actin (Amersham) to check for equal loading of protein in each lane. The ATM antiserum was generated against synthetic peptide close to the NH₂ terminus (residue 819–844; Ref. 19). Using these antibodies, no ATM protein was detected in three cell lines derived from AT patients, who carry mutations leading to truncation downstream from the antibodies binding site. Similar results were obtained by Lakin et al. (31) using different antibodies raised against different segments at the ATM protein. The authors concluded that mutations in AT patients lead to destabilization of ATM mRNA and protein (31).

Solid-Phase Plate RIA. Quantification of the expression of the ATM protein in various B-CLL samples was performed using solid-phase plate RIA. Briefly, the RIA plates were coated overnight at 4°C with 5 µg of protein in 50 µl of PBS. The plates were washed with PBS and blocked with 100 µl of 1% BSA (Amersham) in PBS for 1 h at 37°C. One set of plates was incubated overnight at 4°C with rabbit anti-ATM antibody prediluted 1:250 with PBS containing 0.1% BSA. A second set of plates was incubated under the same conditions with anti-actin antibodies to confirm equal coating of protein. The ATM plates were then washed with PBS and then treated with sheep anti-rabbit (1:500) IgG (Amersham) antisera in 0.1% BSA-PBS for 2 h at 37°C. The actin plates were incubated with rabbit anti-mouse IgM antisera (Jackson Immunology Research Laboratories, West Grove, PA). After they were washed, the plates were developed with excess 125I-labeled protein G (200,000 cpm in 50 µl of 0.1% BSA/PBS per well) for 2 h at room temperature, washed with PBS, and then separated into individual wells that were counted in a gamma counter (Pharmacia LKB Biotechnology, Uppsala, Sweden). Assays were carried out in triplicate, and the results were corrected for nonspecific binding (1–2%) detected in control wells that were not coated with a test antigen but were blocked with BSA.

Results

LOH at the ATM Gene. A first group of 41 paired normal and CLL tumor specimens were screened for LOH with three microsatellite markers located on chromosome 11q13–q23 (Fig. 1); D11S987 is localized near the cyclin D1 gene (32), D11S2179 amplifies a (CA)₉ repeat from intron 34 of the ATM gene (32), and D11S1356 is localized near the MLL gene locus (33). Fluorescent primers were used, and PCR products were analyzed using either the ABI 310 or 373 sequencer (Fig. 2). Five (14%) of 36 informative samples showed LOH with the D11S2179 marker. Two of these five patients exhibited a large deletion encompassing the D11S1356 microsatellite in addition to the D11S2179 microsatellite. No LOH was detected with the D11S987 marker, and none of the tested cases showed LOH in D11S1356 alone without LOH in the D11S2179 marker as well.

Fig. 1. Map of the loci analyzed on chromosome 11.
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Fig. 2. Example of an electropherogram showing LOH at the ATM locus (D11S2179) in B-CLL. Normal tissue (A) and B-CLL tumor sample from the same patient (B) are shown. The numbers on the X-axis refer to data points (not corrected for size), and the numbers on the Y-axis refer to the signal strength.

A homozygous deletion was detected. All analyses were repeated at least twice for confirmation and reproducibility.

Loss of ATM Protein. LOH in a tumor suppressor gene is usually associated with mutation in the second allele. Most of the reported mutations in the ATM gene lead to truncated protein, which is usually associated with instability of the protein, mRNA, or both (35, 36). Lakin et al. (31) and Brown et al. (19) reported lack of ATM protein expression in AT patients with mutations leading to protein truncation. ATM protein was not detected, despite the fact that the truncations were downstream from the antisera binding sites. Therefore, we postulated that there was a lack of ATM expression in those cases of B-CLL that show LOH. We analyzed ATM protein levels in B-CLL tumor samples from patients exhibiting ATM gene deletions (four samples) and compared these levels with levels in samples from B-CLL patients without this deletion (eight samples) as well as in normal samples from patients without any evidence of leukemia (Fig. 3). The samples from the patients with ATM deletion showed an almost complete absence of ATM protein, whereas the samples from B-CLL patients with no ATM gene LOH and the lymphocytes from normal individuals showed a relatively high level of ATM protein.

We also studied another 111 random B-CLL samples for the expression of ATM protein using solid-phase RIA and compared the levels in this group of patients with those in 21 peripheral blood samples. ATM protein levels from five reactive lymph nodes were also compared with those from normal peripheral blood mononuclear cells. The expression of ATM protein in B-CLL patients was compared with the median value seen in normal individuals, which was

Fig. 3. Lack of expression of ATM protein in B-CLL cases with LOH at the ATM gene. Western blot analysis of proteins isolated from four different peripheral blood samples from normal controls (NORMAL), four B-CLL samples with LOH (CLL+LOH), and four B-CLL samples without LOH (CLL) is shown. The filter was stripped and reprobed with β-tubulin antibody to confirm adequate protein loading.

Fig. 4. Boxplots showing the levels of ATM protein as detected by RIA. The median cpm value of the ATM in mononuclear cells from normal individuals was arbitrarily assigned a value of 1. Bars, 95% confidence limits; boxes, 75% of values. Line, median for each group; shaded area, 95% confidence interval; unusual values (outliers) are shown as •.
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Fig. 5. Kaplan-Meier survival curves for all B-CLL patients studied: 43 patients with LOH at the ATM gene or <0.5 ATM protein and 108 patients without LOH or protein deficiency.

arbitrarily assigned a value of 1. Reactive lymph nodes showed ATM protein levels similar to those obtained from peripheral blood mononuclear cells (5–10% variation). Fig. 4 shows the RIA values in boxplot format. The values of the ATM in B-CLL and in mononuclear cells from normal individuals are depicted side by side. The values of actin protein in both groups are shown to confirm that an equal amount of protein was used in the analysis. Of the 111 B-CLL patients, 34% showed ATM levels <0.5 (50% of the normal median); 16 (14%) of these patients showed ATM protein of <0.2. All cases with protein of <0.2 showed no detectable protein by Western blot analysis. The ratios of patients with LOH at the ATM gene and patients with a low level of the ATM protein (≤0.5) were different (14% versus 34%). This apparent discrepancy was perhaps because some patients had mutations in both alleles rather than a deletion in one allele and a mutation in the other. The low level expression of ATM protein (<0.5) is most likely due to the presence of some normal cells in the analyzed samples.

Clinical Data and Correlation with ATM. All patients with B-CLL were diagnosed on the basis of morphology, immunophenotyping, and molecular studies. The median age of the patients was 62 years. All patients were CD5-, CD19-, and CD23-positive and were accepted for the study if CD5- and CD19-positive cells represented >70% of total cells. The morphology of the lymphoid cells in patients with ATM abnormality was not different from those without ATM abnormality. No significant increase in FMC-7 or any unusual expression of surface markers was noted in ATM-deficient patients. Only 9 of the 43 (21%) patients with ATM deficiency (38 by RIA and 5 by RIA and LOH) showed cytogenetic abnormality on chromosome 11 by conventional cytogenetic study. There was no significant difference in sex distribution or levels of lactate dehydrogenase or β2M (P = 0.2) between the two groups. Patients with ATM deficiency had a low platelet count (P = 0.02), lower hemoglobin levels (P = 0.03), and advanced Rai stage (P = 0.03). Patients with ATM deficiency had more aggressive disease and shorter survival times (Fig. 5). Fig. 5 shows the survival probabilities of all ATM-deficient patients (>0.5, ATM patients and LOH patients) compared with patients with normal ATM genes and protein. The difference in survival was significant (P = 0.003). The median survival was 35.6 months for patients with deficient ATM versus 97.3 months for patients with normal ATM. Similar findings were obtained when the survival of the 111 patients studied by protein analysis was evaluated separately from patients with LOH. When patient survival was evaluated according to age (Fig. 6), the impact of ATM deficiency was more striking when patients were younger than 55 years (P = 0.02). In contrast, in patients older than 55 years, survival was not significantly shorter in ATM-deficient patients (Fig. 6). This pattern of correlation with survival is identical to that reported for patients with chromosome 11 deletions (14). Morphological evaluation of bone marrow and peripheral mononuclear cell smears from all patients showed no specific difference between ATM-deficient patients and patients with normal ATM.

Discussion

Although B-CLL is a common leukemia, the disease remains somewhat difficult to manage, and little is known about its molecular basis (5, 6). No objective pathological criteria have been identified to distinguish aggressive disease from slowly progressing disease, and patients with B-CLL may die anywhere from 3 to 15 years after the diagnosis. The recent identification of a deletion on chromosome 13q showed no significant prognostic value for this abnormality (34). However, recent reports demonstrated that abnormalities at 11q22.3–q23.1 define more aggressive disease (14).

We used three microsatellite markers located on chromosome 11 mainly to test whether the ATM gene is involved in these abnormalities. We studied 41 patients for LOH at three genes, ATM, MLL, and BCLI, located around 11q23 and 11q13. The B-CLL patients showed a high incidence (14%) of LOH at the ATM gene and to a lesser degree at the MLL gene, but no LOH at the BCLI gene. The ATM gene is a

Fig. 6. Survival probabilities of B-CLL patients according to age. Top, significantly shorter survival in patients younger than 55 years with ATM deficiency; bottom, no significant difference in survival between patients 55 years or older.
gene. Individuals with heterozygous mutation in the ATM gene partially explain our finding of shorter survival times in B-CLL patients treated for therapy and radiation on B-CLL tumors because these patients have a predisposition for malignancy, particularly CLL (37).

Further studies by Western blot analysis and RIA showed that 34% of 111 cases expressed <50% of the ATM protein detected in normal lymphoid cells. Most of the cases that expressed levels between 20 and 50% showed some dilution of the tumor cells by normal cells (i.e., lymphocytes <90%). The discrepancy between the percentage of LOH at the ATM and lack of expression of ATM by protein may reflect the possibility that some cases have mutations in both alleles rather than a deletion in one allele and a mutation in the second. It is also possible that low expression of ATM in these cases is due to altered methylation pattern of the gene’s regulatory sequences. Upon reviewing the morphology and phenotype of the cases, we observed no specific characteristics in B-CLL cases with abnormality at the ATM gene. However, we demonstrated a significantly shorter survival time in patients with ATM deficiency ($P = 0.003$). Furthermore, we demonstrated that ATM deficiency is an important prognostic factor in patients younger than 55 in a fashion similar to that reported for patients with chromosome 11 deletions (14). We also found that ATM deficiency correlates with a more advanced Rai stage ($P = 0.03$), lower hemoglobin level ($P = 0.03$), and lower platelet count ($P = 0.02$). It did not significantly correlate with $\beta_2 M (P = 0.2)$. $\beta_2 M$ results were available for only 69 patients, and perhaps the number of patients is too small to draw a definite conclusion regarding a correlation with ATM deficiency.

The ATM gene product belongs to a family of proteins that are involved in cell cycle control, DNA repair, and recombination. The ATM protein is critical for both cellular response to ionization radiation and normal cell cycle progression (20, 21). Furthermore, the ATM gene is implicated in lymphoproliferative disease because the ATM-deficient mice develop lymphomas, and our data suggest that the ATM gene is probably the gene that is important in the 1q22–23 abnormality seen in B-CLL. However, this needs to be confirmed by demonstrating mutation in the other allele in patients with LOH at the ATM gene. Individuals with heterozygous mutation in the ATM gene have an increased incidence of malignancy, particularly CLL (37). Although not discussed here, our data indicate that some of our patients with ATM deficiency have germ-line mutations in the ATM gene. ATM heterozygous mutation is expected to be around 1% in the general population, and perhaps the reported familial clustering of CLL results from ATM germ-line mutation in these families (38, 39).

The ATM protein abnormalities in B-CLL also raise questions regarding the treatment of these patients, particularly the effects of chemotherapy and radiation on B-CLL tumors because these patients have a higher sensitivity to ionizing radiation. This complication may partially explain our finding of shorter survival times in B-CLL patients with ATM deficiency.

References


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