Incidence and Clinical Implications of ATM Aberrations in Chronic Lymphocytic Leukemia

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A subset of chronic lymphocytic leukemia (CLL) carries mutations in ataxia telangiectasia mutated (*ATM*). Such *ATM* mutations may be particularly relevant in the setting of del11q, which invariably results in the deletion of one *ATM* allele. To improve our understanding of the frequency and type of *ATM* mutations that exist in CLL, we resequenced all *ATM* coding exons in 24 CLL with del11q using direct sequencing. We detected two missense mutations, resulting in an *ATM* mutation frequency of 8%; nonsense and frameshift mutations were not identified. Given the low *ATM* mutation frequency detected in this cohort, we proceeded with measurements of nonmutational ATM aberrations in CLL through analysis of the activation state of ATM in response to external irradiation. The phosphorylation state of ATM at Ser-1981 was measured using quantitative immunoblotting in purified CLL cells isolated from 251 CLL patients; data were normalized to simultaneous measurements of total ATM protein and actin. Resulting p-ATM/ATM and p-ATM/actin ratios were subsequently analyzed for prognostic significance inclusive and exclusive of *TP53* exons 2–10 mutations. From these analyses, conducted in a large prospectively enrolled CLL patient cohort, neither the p-ATM/ATM nor the p-ATM/actin ratios were found to be prognostic for short survival. These data in aggregate demonstrate a low frequency of ATM aberrations in an unselected CLL cohort and do not support a major prognostic role for ATM aberrations in CLL, thus motivating renewed research efforts aimed at understanding the pathobiology of 11q deletions in CLL.

INTRODUCTION

The pathogenesis of chronic lymphocytic leukemia (CLL) remains incompletely understood (Chiorazzi and Ferrarini, 2011). Consequently, further improvements in understanding the biology and determinants of clinical outcome of CLL rely on investigations into recurrent molecular aberrations and their effects on clinical disease characteristics. Genomic aberrations are a CLL trait that is of fundamental importance to CLL biology and clinical outcome (Juliusson et al., 1990; Fegan et al., 1995; Dohner et al., 2000; Shanafelt et al., 2008; Ouillette et al., 2011). One of the most frequent recurrent genomic aberrations in CLL is deletion 11q (del11q), which comprises a heterogeneous set of mostly large interstitial deletions that are frequently many megabases in length (Ouillette et al., 2010). All CLL-associated 11q deletions result in the removal of one ataxia telangiectasia mutated (*ATM*) allele, and this almost always occurs in the context of a large number of co-deleted genes. As *ATM* is recurrently mutated in CLL, it has attracted attention as one of the genes contributing to 11q biology (Bullrich et al., 1999; Schaffner et al., 1999; Stankovic et al., 1999).

Given that ATM is a very large gene with >60 coding exons, unbiased estimates of the frequency of somatically acquired *ATM* mutations in CLL are sparse. Furthermore, lack of analysis of paired normal DNA in some studies may have resulted in the identification of germline variants of unclear pathogenetic relevance as opposed to somatic variants. Here, we combined *ATM* sequence analysis with a functional ATM assay to derive unbiased estimates of aberrant ATM states in a large CLL cohort. Our data in summary allow for the conclusion that aberrant ATM states in CLL are infrequent and not associated with substantially shortened survival.

Additional Supporting Information may be found in the online version of this article.

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METHODS

Patients

This study is based on a prospectively enrolled CLL patient cohort as described (Ouillette et al., 2011). The trial was approved by the University of Michigan Institutional Review Board (IRBMED #2004-0962) and written informed consent was obtained from all patients before enrollment.

ATM Sequence Analysis and CLL fluorescence in-situ hybridization (FISH) Analysis

Sequence analysis of all 62 *ATM* coding exons was performed using direct sequencing of polymerase chain reaction (PCR) amplicons, which were derived from DNA isolated from flow cytometer-sorted CD19+ cells cryopreserved at the time of study enrollment. The somatic nature of mutations was confirmed using paired template DNA isolated from flow cytometer-sorted CD3+ cells. Exon sequence coverage exceeded 99% through use, where needed, of multiple primers per exon.

The 11q status of all CLL cases was determined at the Mayo Clinic, Rochester, MN, as part of routine clinical CLL FISH testing. Probes used were located at D11Z1 for 11cen and *ATM* (Abbott Laboratories, Des Plaines, IL, Vysis LSI ATM SpectrumOrange probe, $\sim$ 500 kilobases in length spanning the entire ATM gene plus adjacent genes from $\sim$ D11S1826 to D11S1294) in 11q22.3 with $<5\%$ as the cutoff for normal and with 200 interphase nuclei counted per probe. The 11q status was also assessed using single-nucleotide polymorphism (SNP) 6.0 arrays as published (Ouillette et al., 2011).

Measurements of Normalized ATM Expression Using Q-PCR

RNA was prepared from $\sim 2 \times 10^5$–10^6 ultrapure CD19+ flow cytometer-sorted cells using the Trizol reagent and resuspended in 50 μL diethylpyrocarbonate-treated water. Complementary DNA was made from $\sim$ 20 ng of RNA using the Superscript III first strand synthesis kit (Invitrogen, Carlsbad, CA) and oligo-dT priming. Primers and TaqMan-based probes were purchased from Applied Biosystems, Carlsbad, CA (ATM probe Hs01112307_m1). Duplicate amplification reactions included primers/probes, TaqMan® 2× Universal PCR Master Mix, No AmpErase uracil-N-glycosylase and 1 μL of cDNA in a 20 μL reaction volume. Normalization of relative copy number estimates for ATM RNA was done with the Ct values for PGK1 as reference (delta Ct mean ATM minus CT mean PGK1).

Measurements of ATM Gene Methylation using the HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) Assay

HELP assays were used to study methylation using a published standard protocol (Shaknovich et al., 2010). We digested 500 ng of high molecular weight DNA using HpaII and MspI (NEB, Ipswich, MA). This was followed by adaptor ligation using T4 DNA ligase and PCR amplification favoring 200–2000 bp products and labeling of products. Amplified products were cohybridized to custom NimbleGen HELP microarrays (NimbleGen, Inc. Madison, WI). Primary data processing was performed using a HELP pipeline with minor modifications (Thompson et al., 2008). Post pipeline data were quartile normalized for intra- and interarray normalization and the quartile-normalized value ($\log_2$(HpaII/MspI)) was used in all calculations as a measurement of the methylation state of individual interrogated genomic loci. The ($\log_2$(HpaII/MspI)) is inversely proportional to the degree of methylation, so 1/($\log_2$(HpaII/MspI)) was used in the graphics for clarity.

Measurements of Radiation-Induced ATM Autophosphorylation in CLL

These measurements were performed as described (Ouillette et al., 2010). We extended previously published quantitative immunoblot data on the induced phosphorylation state of ATM at Ser-1981 that follows external CLL cell irradiation ($N = 141$) to an expanded CLL cohort (data on a combined $N = 251$, for which sufficient cryopreserved specimens were available, are reported here; Bakkenist and Kastan, 2003).

Briefly, cryopreserved CLL cells were thawed, washed, and depleted of CD3- and CD14-positive cells using negative selection over LS columns (Miltenyi Biotec, Auburn, CA). Tumor cells thus purified were aliquoted into low-cell-binding tissue culture plates (Nunc, Waltham, MA, #145385) at concentrations of 1.2×10^7 per milliliter. Cells rested for 1–1.5 h before treatment with 5 Gy of ionizing radiation (Philips 250 kV X-ray–emitting source). Cells for Western blot were harvested 20 min after irradiation treatment, washed once with 1× phosphate-buffered saline,

TABLE 1. Results of Genomic DNA-based Exon Resequencing of *ATM* in CLL Cases with del11q and Corresponding pATM/ATM, pATM/actin, and Total ATM/actin Ratios

CLL no.	Type of sequence variants	Sequence variants description (based on NM_000051.3)	pATM/ATM ratio	pATM/actin ratio	Total ATM/actin ratio
230	Germline missense	c.370A>G, p.124 I>V	1	0.41	0.41
24	Germline intronic	A>C 6bp upstream of exon 8	1.03	0.07	0.07
240	Nonsynonymous SNP	SNP rs61734354, A>G, 870N>D	1.27	0.61	0.48
227	Nonsynonymous SNP	SNP rs56398245, A>G, 1128Q>R	0.75	0.3	0.40
230	Nonsynonymous SNP	SNP rs3092856, C>T, 1380H>Y	1	0.41	0.41
101	Nonsynonymous SNP	SNP rs1801516, G>A, 1853D>N	0.46	0.1	0.21
173	Somatic missense	c.7184A>T, p.2395 D>V	0.69	0.08	0.12
226	Somatic missense	c.7213A>T, p.2405M>L	0.65	0.42	0.64

and lysed on ice for 20 min with lysis buffer (50 mmol/L Tris, 100 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, 1% Triton X-100, and 20 mmol/L NaF) supplemented with fresh protease and phosphatase inhibitors (1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× Sigma-Aldrich (St. Louis, MO) protease and phosphatase inhibitor cocktails). Western blots were carried out for both irradiated and nonirradiated CLL-derived tumor samples for phospho-ATM (Ser1981, Rockland Immunochemicals, Boyertown, PA), total ATM (Santa Cruz Biotech, Santa Cruz, CA), actin (Sigma-Aldrich, St. Louis, MO), and alpha-tubulin (Santa Cruz) for all patients. All Western blots included two pooled standard samples and uniform cell equivalent loading to enable quantitation across multiple blots.

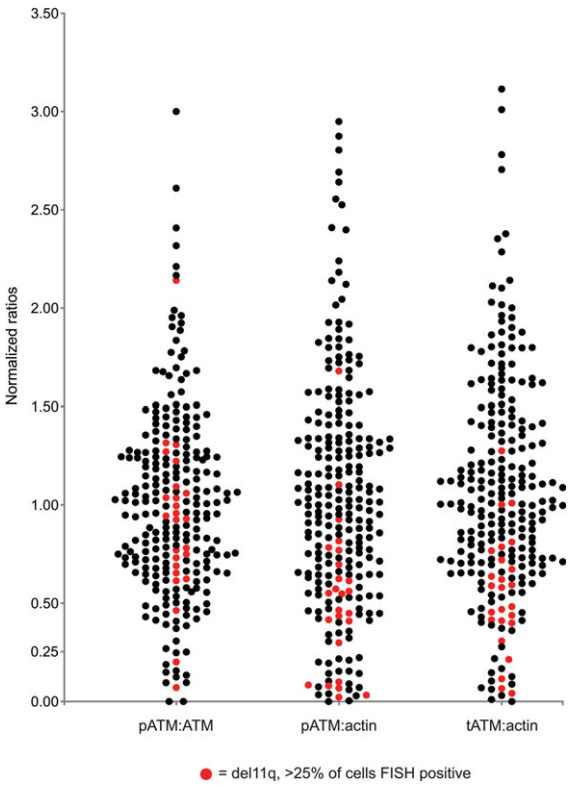

Figure 1. Display of pATM/ATM, pATM/actin, and total ATM/actin ratios. Data are based on normalized quantitative immunoblotting of cell lysates made from purified CLL cells 20 min after 5 Gy external irradiation as described in Ouillette et al., (2010) ($N = 250$, of which 141 were previously published but are included here for completeness).

CLL Outcome Analysis

Outcome analysis included all CLL (up to serial enrollment #266 and exclusive of enrollment failures and samples without sufficient cryopreserved specimens) for which complete characterization and mature survival data were available (Ouillette et al., 2011). To obtain as complete a result as possible, we conducted analyses based on: (i) dichotomized CLL cohorts using pATM/ATM or pATM/actin ratios of <0.5 or <0.25 as thresholds [in quantitative normalized immunoblotting, a ratio range of 0.5–2.0 likely represents assay variations and as such only values below 0.5 or alternatively 0.25 (more stringent) were considered abnormal]; (ii) ratios organized by pATM/ATM quartiles; (iii) the CLL cohort that was untreated at the date of trial enrollment and using the trial enrollment date (equal to the sample procurement date) as the reference date for analysis; (iv) the CLL cohort that was untreated at the date of trial enrollment and using the CLL diagnosis date as the reference date for analysis; or (v) all CLL analyzed independent of treatment status at enrollment. Furthermore, analyses were performed in parallel in the cohorts after exclusion of all cases carrying *TP53* exon 2–10 mutations, or del11q, or both.

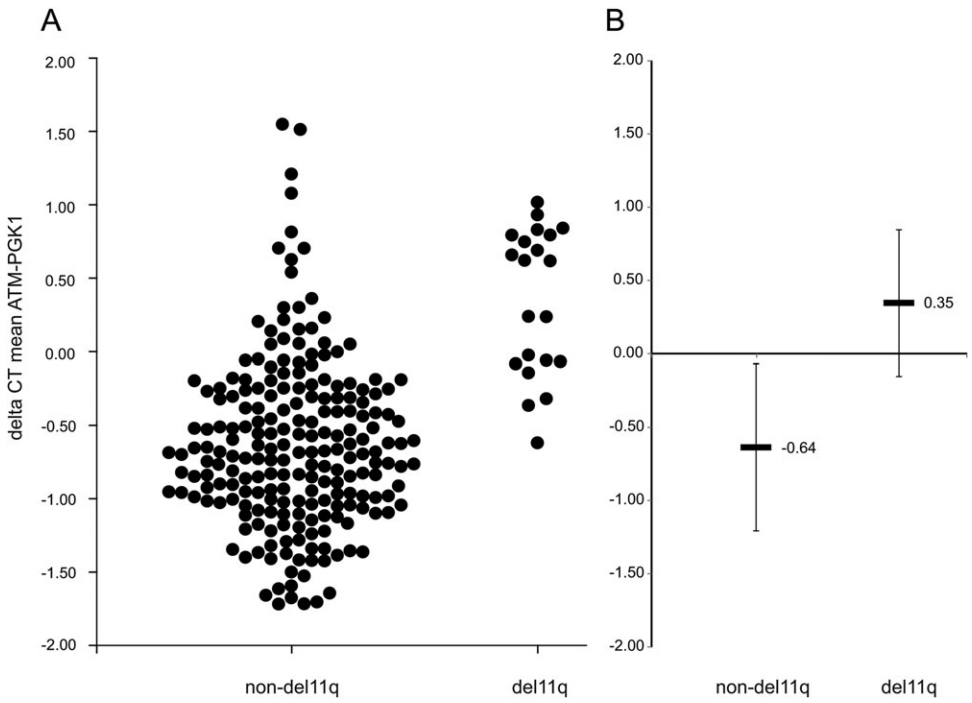

Figure 2. Display of Q-PCR results for ATM and PGK1 (delta Ct mean ATM-PGK1 values) grouped by 11q status. Panel A: display of delta Ct mean ATM-PGK1 values. Each dot represents the mean of duplicate measurements for a patient. Panel B: display of the mean of all delta Ct mean ATM-PGK1 values plus 2SD. Samples are grouped by 11q status.

Clinical outcome analysis followed previously published definitions and methods (Ouillette et al., 2011). Time to subsequent therapy (TTST) was calculated for all patients who received at least one chemotherapy treatment regimen and was either based on the actual start date of subsequent therapy or on a censor date.

RESULTS

To complement existing knowledge of the frequency and clinical impact of ATM aberrations in CLL and to provide guidance and stimulus for future research on 11q deletions, we determined the frequency of somatically acquired *ATM* mutations in 24 CLL cases that carried del11q. Twenty-three of these cases comprised those with del11q (present in $\geq$25% of nuclei by FISH and positive by SNP 6.0 array profiling, see Supporting Information, Table 1) that are part of a well-characterized, prospectively enrolled cohort of 255 CLL patients for which template DNA and mature clinical outcome data are available (Ouillette et al., 2011).

A total of two *ATM* mutated CLL cases were identified, resulting in a mutation frequency estimate of 8% in this subset of CLL with del11q. Both these *ATM* mutations were missense mutations; nonsense mutations or frameshift mutations were not identified. In addition, four cases carried nonsynonymous SNPs and one case a missense germline mutation; each converted to homozygosity as a consequence of del11q. One additional case displayed an intronic SNP located six basepairs from the relevant exon start site. Of these eight cases with *ATM* sequence variants, three cases displayed aberrant phosphorylated ATM (p-ATM) to actin ratios of <10% of wildtype values (see below) and four additional cases displayed p-ATM/actin ratios of <0.5. The pATM/ATM ratio was within 0.46–1.27 for all cases. Data are summarized in Table 1.

Next, we extended previously published quantitative immunoblot data on the induced phosphorylation state of ATM at Ser-1981 that follows external CLL cell irradiation ($N = 141$) to all cases from an expanded CLL cohort for which sufficient cryopreserved cells were available (data on a combined $N = 251$ are reported here, see Supporting Information, Table 1) using the published experimental protocol (Ouillette et al., 2010). Normalized p-ATM measurements were divided by normalized total ATM or actin measurements, resulting in p-ATM/ATM ratios (a

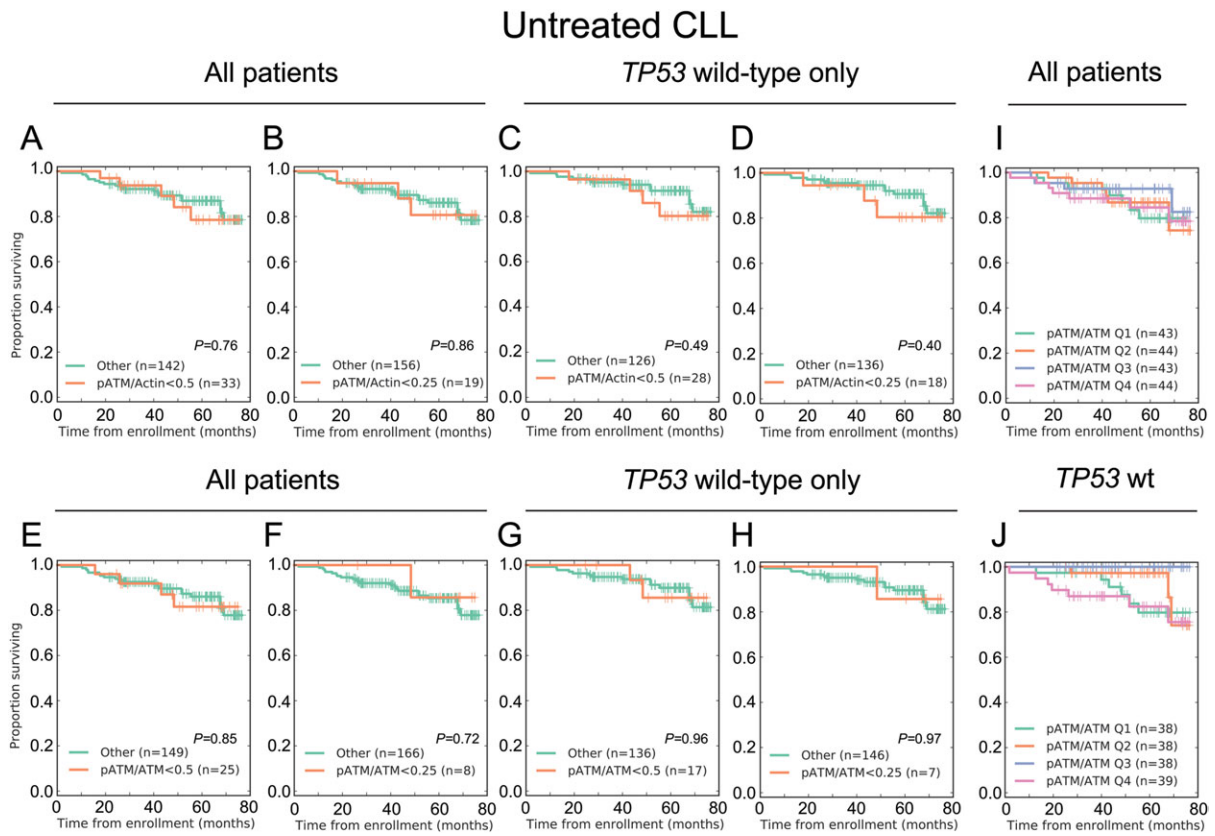

Figure 3. Results of outcome analysis for CLL cohorts categorized based on pATM/ATM or pATM/actin ratios. Only untreated patients, analyzed from date of trial enrolled. Panels I and J: quartile analysis, with Q1 representing the lowest ratios and Q4 the highest.

measure of the activation state of ATM molecules) and p-ATM/actin ratios (a measure of the activation state of ATM protein relative to an unrelated protein). Furthermore, we generated total ATM/actin ratios to detect aberrantly low total ATM protein expression (Starostik et al., 1998). Normalized ratios are displayed in Figure 1. CLL cases with 11q deletions present in ≥25 of interphase nuclei are marked in red.

Review of the aforementioned data allowed for the following important conclusions to be drawn: (i) the vast majority of CLL (∼ 87%) are characterized by normal ATM protein levels that are associated with normal ATM activation after external radiation (pATM/ATM ratio ≥0.5), and this fraction increases to 95% for the more stringent cutoff of pATM/ATM ratio ≥0.25; (ii) only two CLL cases with del11q displayed aberrantly low p-ATM/ATM ratios, indicating that following DNA damage the ATM protein was appropriately activated in 90% of CLL with del11q; (iii) within the group of 11q-deleted CLL, a subset displayed aberrantly low pATM/actin and total ATM/actin ratios, together indicating relatively low ATM protein expression; and (iv) a small subset of the analyzed CLL cases without del11q displayed either low ATM activation or low ATM protein.

Given the latter observations, we reasoned that low or absent ATM mRNA may account for the few CLL cases with low ATM protein expression. We therefore proceeded with measurements of ATM mRNA through Q-PCR. The data were subsequently grouped by 11q status (del11q was present in ≥25% of nuclei) and are displayed in Figures 2A and 2B. Review of this data allowed for the following conclusions: (i) all CLL cases express ATM mRNA and overall at rather uniform levels, and therefore absence of ATM mRNA is not the reason for low ATM protein; (ii) CLL cases with del11q display a gene dosage effect resulting in twofold lower ATM mRNA that appears insufficient to explain low to absent ATM protein in the few relevant cases (for the CLL cases with tATM/actin ratios of 0–0.49 the mean delta Ct ATM-PGK1 value was −0.4 and for cases with tATM/actin ratios of ≥0.5 the mean delta Ct ATM-PGK1 value was −0.6). Therefore, it is hypothesized that

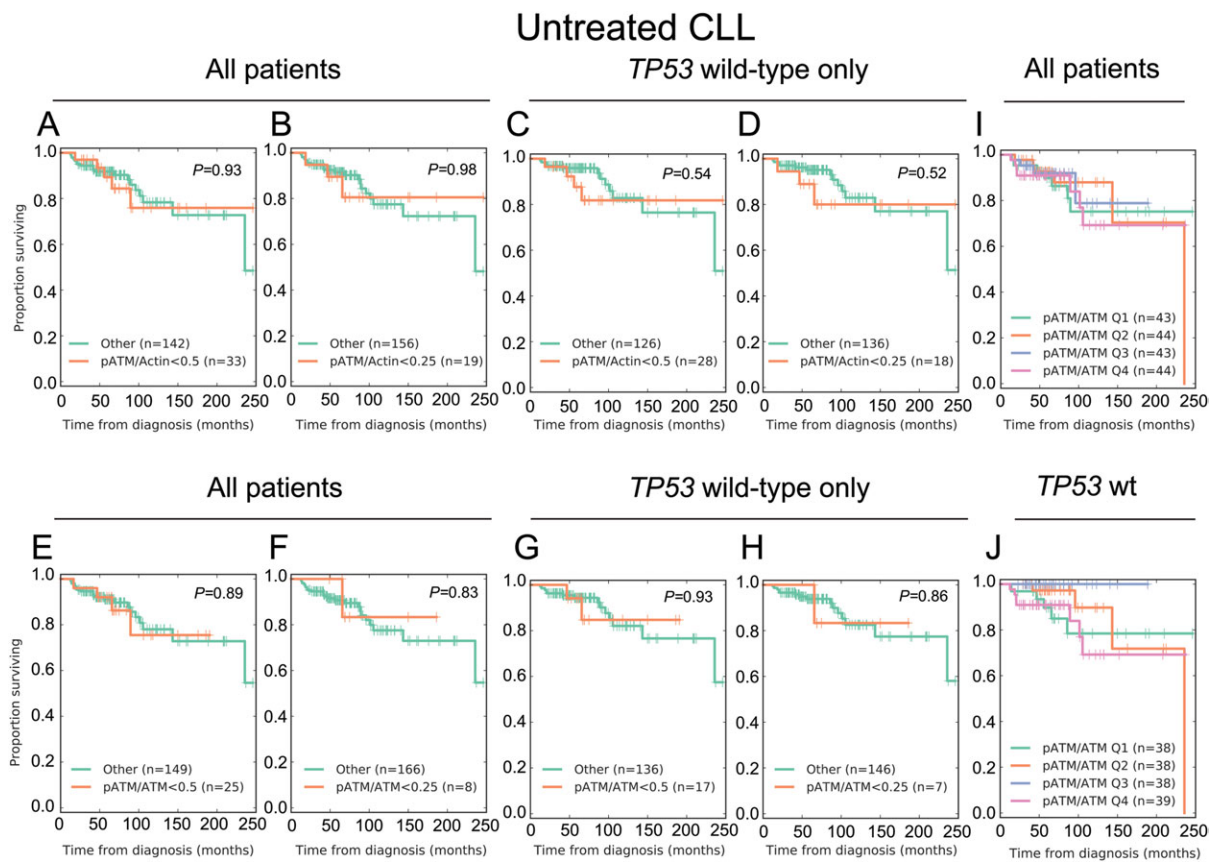

Figure 4. Results of outcome analysis for CLL cohorts categorized based on pATM/ATM or pATM/actin ratios. Only untreated patients, analyzed from date of diagnosis. Panels I and J: quartile analysis, with Q1 representing the lowest ratios and Q4 the highest.

posttranscriptional regulation of ATM mRNA or protein is operational in a small subset of CLL, resulting in depressed total ATM protein levels.

We also analyzed the *ATM* gene for methylation events using the HELP assay (see methods). Data from 246 CLL cases were compared with results for a small number of nonmalignant B-cells. In total, five distinct genomic regions were interrogated, with three of them located in the *ATM* promoter region. These data in aggregate demonstrate that the methylation status of the *ATM* gene is different from the status of nonmalignant B-cells (see Supporting Information, Figure 1). Next, we wished to determine if the degree of any of the aberrant ATM methylation sites correlated with the Q-PCR-based assessments of ATM mRNA levels. No such correlations were found.

Next, we determined effects of aberrant ATM protein activation (pATM/ATM ratios) or the amount of activated ATM relative to an unrelated protein (pATM/actin ratios) on clinical outcome in CLL using the variables TTST and overall survival (OS). Data are summarized in Figures 3 and 4 and in Supporting Information, Figures 2 and 3 for OS and Figures 4–7 for TTST.

Review of the TTST analysis performed in all patients and in various subcohorts (all patients or patients without *TP53* mutations, without del11q or without either *TP53* mutations or del11q) suggested shortened TTST as a function of aberrant ATM (most notably for the analysis of p-ATM/actin ratios).

Review of the OS analysis performed in all patients or the subcohort without *TP53* mutations demonstrated no effect of aberrant ATM on survival, thus suggesting effective salvage after frontline treatment. In comparison, TTST and OS analysis for *TP53* mutated CLL demonstrated expected strong negative effects (Supporting Information, Figure 8).

DISCUSSION

In this study, we provide important novel data on the frequency of ATM aberrations in

CLL. These data constitute the most complete assessment of ATM aberrations in CLL published today and uniquely incorporate a functional assessment of ATM into the overall analysis. Using direct sequencing of PCR amplicons templated on DNA isolated from flow cytometer-sorted CD19+ cells and confirmed with DNA isolated from CD3+ T-cells, we find a lower frequency of somatically acquired *ATM* mutations in CLL with del11q than previously reported, although our findings are in line with aggregate data derived from large-scale kineome, exome, or whole-genome sequencing studies and clinical studies (Austen et al., 2005; Quesada et al., 2011; Wang et al., 2011; Zhang et al., 2011; Lozanski et al., 2012). In this cohort, we did not detect *ATM* mutations that are usually indicative of a tumor suppressor function (mutations resulting in aberrantly truncated or altered proteins). Albeit rarely such mutations have been previously identified (Yuille et al., 2002).

Using quantitative immunoblotting on a very large CLL cohort, we did detect CLL cases with low ATM protein expression (Starostik et al., 1998). As these cases were not associated with loss of ATM mRNA expression, we hypothesize that ATM mRNA translation or ATM protein stability is impaired in these cases. This may be deserving of further study.

Importantly, based on this comprehensive interrogation of *ATM* mutations, ATM activation, and ATM expression in a large prospectively collected CLL cohort, we could not detect frequent aberrations in ATM or a strong negative effect of aberrantly low ATM activation or low total ATM protein expression on CLL survival (Austen et al., 2007). These data thus strengthen the rationale to search for and prospectively validate other del11q-associated molecular defects that could account for the relatively distinct clinical course of these patients (Herling et al., 2009; Gaudio et al., 2011; Saiya-Cork et al., 2011) and may be used to generate hypotheses to explain the apparent relative improvements in outcome of CLL patients harboring 11q deletions treated with chemoimmunotherapy.

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