## ORIGINAL ARTICLE

# ATM function and its relationship with *ATM* gene mutations in chronic lymphocytic leukemia with the recurrent deletion (11q22.3-23.2)

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Approximately 10–20% of chronic lymphocytic leukemia (CLL) patients exhibit del(11q22–23) before treatment, this cohort increases to over 40% upon progression following chemoimmunotherapy. The coding sequence of the DNA damage response gene, ataxia-telangiectasia-mutated (*ATM*), is contained in this deletion. The residual *ATM* allele is frequently mutated, suggesting a relationship between gene function and clinical response. To investigate this possibility, we sought to develop and validate an assay for the function of ATM protein in these patients. SMC1 (structural maintenance of chromosomes 1) and KAP1 (KRAB-associated protein 1) were found to be unique substrates of ATM kinase by immunoblot detection following ionizing radiation. Using a pool of eight fluorescence *in situ* hybridization-negative CLL samples as a standard, the phosphorylation of SMC1 and KAP1 from 46 del (11q22–23) samples was analyzed using normal mixture model-based clustering. This identified 13 samples (28%) that were deficient in ATM function. Targeted sequencing of the *ATM* gene of these samples, with reference to genomic DNA, revealed 12 somatic mutations and 15 germline mutations in these samples. No strong correlation was observed between *ATM* mutation and function. Therefore, mutation status may not be taken as an indicator of ATM function. Rather, a direct assay of the kinase activity should be used in the development of therapies.

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## INTRODUCTION

Recurrent cytogenetic abnormalities occur frequently in chronic lymphocytic leukemia (CLL), ~ 70–80% of cases exhibit recurrent chromosomal abnormalities that can be identified by fluorescence *in situ* hybridization (FISH). The most common genomic aberrations, which include deletion at 13q, 11q, 17p and 6q, as well as a trisomy of chromosome 12, were assigned prognostic values.[1] Approximately 10–20% of previously untreated patients with CLL exhibit a substantial deletion in the q arm of chromosome 11, the site of the ataxia-telangiectasia-mutated (*ATM*) gene at (11q22.3), which is within the minimally deleted region.[2,3] The ATM protein is an important regulator of the DNA damage response pathway; it is therefore notable that deletion of 11q has been associated with resistance to DNA-damaging chemotherapy.[4] Despite the strong prognostic significance of FISH analysis for 11q deletion, mutations in *ATM* may also provide clinically vital information. Regarding 11q22–23 deletion (hereafter referred to as 11q deletion), ~ 30–40% of such cases have been reported to have a mutation in the remaining *ATM* allele.[5] Although neither 11q deletion nor *ATM* mutation leads to full loss of p53 function and complete chemoresistance,[5,6] the combination of 11q deletion and *ATM* mutation is significantly associated with impaired responses to alkylating agents and purine analogs.[7] However, because the pathogenic potential of most missense mutations in the *ATM* gene are not known, sequencing alone may not be a reliable predictor of ATM function. Therefore, assays of

ATM function might complement clinically relevant information in addition to that from FISH and *ATM* mutation analysis.

Several different approaches have been applied to assess ATM protein function in CLL cells. For instance, the functionality of ATM has been assessed through reverse transcription-PCR tests of the upregulation of a selected number of *ATM/p53* target genes in response to DNA damage.[8] In addition, measuring damage-induced apoptosis *in vitro* by cytotoxicity assays has also provided information regarding ATM function. For example, sensitive *ATM* mutant and resistant *TP53* mutant tumors responded differently to etoposide in the presence of MDM2 inhibitors (nutlins).[9] Furthermore, the cytotoxic response to doxorubicin is capable of distinguishing between *ATM* mutant and *ATM* wild-type tumors.[10] These assays are limited by their reliance on end points that are not direct *ATM* substrates. A more direct approach involves the measurement of the phosphorylation of proteins that are specific substrates of the ATM kinase such as autophosphorylation of ATM or the phosphorylation of SMC1 (structural maintenance of chromosomes 1), Nbs1 and p53 following treatment with ionizing radiation (IR).[5,6] However, because of the complexity in the double-strand break (DSB) response, not all *ATM* substrates exert the same degree of dependence upon ATM.

In the present investigation, we sought to integrate prior efforts to establish a biomarker for ATM function in CLL cells exhibiting deletion 11q by validating an assay of ATM activity and seeking associations with mutations in the *ATM* gene determined by next-generation sequencing.

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## MATERIALS AND METHODS

### Cell lines
GM16666 and GM16667, obtained from the Coriell Cell Repository (Coriell Institute of Medical Research, Camden, NJ, USA) and cultured in Dulbecco's modified Eagle's medium with high glucose and 20% fetal bovine serum, are matched lines derived from the AT22IJE-T A–T cell line, a fibroblast cell line originated from an ataxia-telangiectasia patient in which ATM protein is undetectable owing to a homozygous frameshift mutation at codon of 762 in the *ATM* gene.[11] It was transfected with either an ATM expression construct (GM16667) or an empty vector (GM16666) and maintained under hygromycin selection to generate A–T-corrected and A–T-deficient stable cell lines.[12]

### CLL samples
Peripheral blood samples from 54 patients with CLL were used in this study. They were collected from patients who consented to the Declaration of Helsinki guidelines through the institutional review board–approved protocols. Peripheral blood mononuclear cells were isolated from leukemic-phase blood as described previously,[13] and the cells were maintained at $10^7$ cells per ml concentration at 37 °C overnight or 1 h before 10 Gy irradiation with Gamma Cell 1000 irradiator (Best Theratronics, Ottawa, ON, Canada). The characteristics of 46 CLL samples in which 11q deletion was identified by fluorescent *in situ* hybridization are described in the Supplementary Table 1.

### Chemicals and antibodies
Inhibitors of DNA-PK ((DNA-dependent protein kinase; NU-7441; Calbiochem, currently Millipore, Billerica, MA, USA), ATM (KU60019; AstraZeneca, Wilmington, DE, USA) and ATR (VE-821; Selleck Chemicals, Houston, TX, USA) was stored in dimethyl sulfoxide at − 80 °C. The DSB-inducing agents neocarzinostatin, etoposide and doxorubicin were all from Sigma-Aldrich (St Louis, MO, USA).

Source of antibodies are as follows: ATM phospho-Ser1981 and ATM (Millipore), SMC1 and SMC1 phospho-Ser966 (Abcam, Cambridge, MA, USA), KAP1 (KRAB-associated protein 1; BD Biosciences, San Jose, CA, USA), KAP1 phospho-Ser824 (Bethyl Laboratories, Montgomery, TX, USA) and β-actin (Sigma-Aldrich). IR DYE 680RD-conjugated goat anti-mouse immunoglobulin G and IR DYE 800CW-conjugated goat anti-rabbit immunoglobulin G (LI-COR, Lincoln, NE, USA).

### Immunoblotting
Cellular lysates were prepared and immunoblot analysis and visualization using a LI-COR Odyssey Infrared Imager (Lincoln, NE, USA) was performed as described previously.[13] Quantitation analysis was carried out by Image Studio software (LI-COR).

### Statistical analysis
The proportions of pSMC1 to SMC and pKAP1 to KAP were taken into account when determining the function of ATM. The normal mixture model-based clustering method was used to identify groups of samples that lost ATM function in this study[14] and details were described in the Supplementary Methods.

### Targeted sequencing of the *ATM* gene
T cells from frozen CLL patient samples were isolated and expanded using Dynabeads Human T Expander CD3/28 according to the manufacturer's instruction (Life Technology, Grand Island, NY, USA). Genomic DNAs from T cells and CLL cells were obtained using a QIAamp DNA Extraction Kit (Qiagen, Valencia, CA, USA). Primers were designed to cover all 66 exons to selectively capture and amplify the *ATM* genomic DNA sequence (SeqCap EZ Choice Library; Roche NimbleGen, Madison, WI, USA). The sequencing was performed using the Illumina Hi-Seq sequencing system in the UT MD Anderson Cancer Center DNA Analysis Core Facility (Houston, TX, USA).

### Somatic mutation detection from captured DNA sequencing
The raw paired-end reads in FASTQ format were aligned to the human reference genome (hg19), using MOSAIK[15] alignment software. The exonic regions in ATM had about 400 × mean depth of coverage. The resulting alignments were analyzed with PCR duplicate removal using the Bayesian model-based software GigaBayes/FreeBayes[16] (details are described in the Supplementary Methods).

### Methylation analysis with pyrosequencing
Both bisulfite conversion and subsequent pyrosequencing analysis were carried out as described in Supplementary Methods at the DNA Methylation Analysis Core (MD Anderson Cancer Center).

## RESULTS

### Identification of ATM substrates
Numerous proteins are substrates for the ATM kinase.[17] Among them, SMC1, a member of the chromosome structure maintenance family, has been demonstrated to be an ATM-specific substrate (Ser966) in response to ionizing irradiation.[18] KAP1 (TRIM28), a transcriptional repressor, is another ATM downstream target that is phosphorylated at Ser824 after IR treatment.[19] Because these two sites have been reported to be specifically phosphorylated by ATM, we chose to validate them as indicators of ATM function. To this end, human fibroblasts from an A–T patient that had been complemented with *ATM* cDNA were irradiated (10 Gy), lysates were prepared 1 h later and protein phosphorylation was analyzed by immunoblotting (Figure 1a). Both KAP1 and SMC1 were phosphorylated in response to IR, but this was greatly reduced by an inhibitor of ATM kinase. In contrast, neither an inhibitor of ATR nor of DNA-PK diminished the IR-induced phosphorylation of these proteins. In addition, SMC1 and KAP1 were minimally phosphorylated in the parental ATM-null cells treated with IR (data not shown). These results indicate that SMC1 and KAP1 are ATM-specific substrates in response to IR.

### Phosphorylation of SMC1 and KAP1 is proportional to ATM protein
To further determine whether phosphorylation of SMC1 and KAP1 are linearly correlated with ATM protein level, A–T-null cells and A–T-repleted cells were mixed at the percentage proportions of 100:0, 75:25, 50:50, 25:75 and 0:100 based on cell numbers. These cell mixtures were then subjected to 10 Gy of irradiation and allowed to recover at 37 °C for 1 h before lysates were prepared. The phosphorylation levels of SMC1 and KAP1 in the mixtures were examined by immunoblots in triplicates in four independent experiments and quantitated by the Li-Cor software (Supplementary Figure 1A). As shown in Figure 1b, SMC1 and KAP1 phosphorylation increased in proportion to the amount of ATM protein. SMC1 and KAP1 phosphorylation each exhibits a linear correlation (5–75%) with the percentage of ATM amount (Supplementary Figure 1B). To determine whether the function of ATM in a cell population could be quantitated based on the phosphorylation of KAP1 and SMC1 induced by other DNA-damaging agents, we investigated the effects on SMC1 and KAP1 phosphorylation after exposure of cells to neocarzinostatin, etoposide and doxorubicin, all of which induce double-strand DNA breaks.[9,10,20,21] As described above, A–T cell mixtures were subjected to the drug treatment in place of IR, and the phosphorylation levels of SMC1 and KAP1 were quantitated to assess ATM function. However, none of these agents triggered a linear increase of SMC1 and KAP1 phosphorylation as the ATM protein level increased (Supplementary Figure 2). Based on these observations, we concluded the phosphorylation of SMC1 and KAP1 after IR was the most accurate indicator of ATM function.

### ATM function in 11q deletion CLL samples
To establish a standard for this assay, we collected samples from eight CLL patients with normal FISH karyotypes. These samples of live cells were mock treated or irradiated (10 Gy) and protein lysates were harvested after 1 h recovery at 37 °C. The individual lysates and a Pool of equal portions of each lysate were then
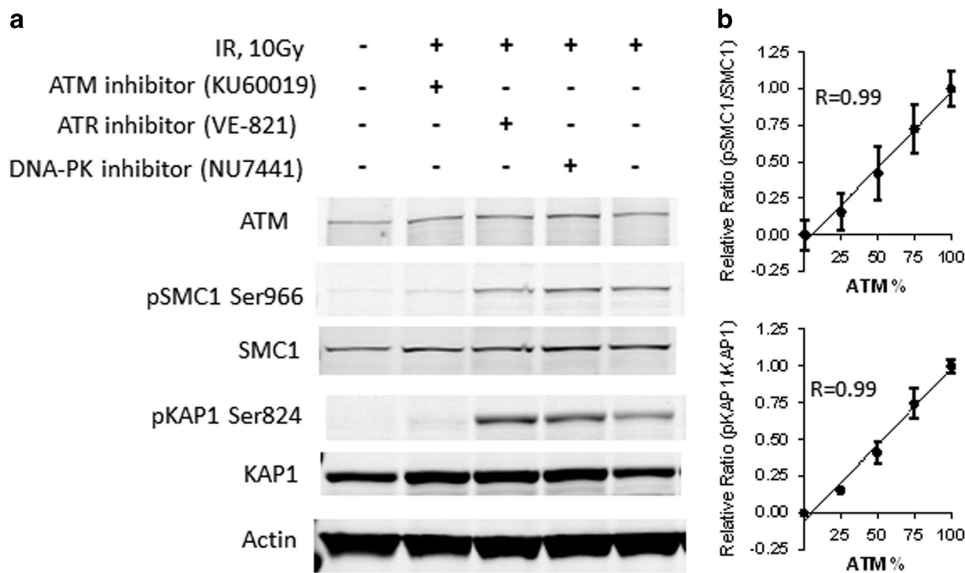
**Figure 1.** Identification of ATM substrates. (**a**) ATM-repleted (GM16667) cells were pre-treated with inhibitors of ATM, ATR and DNA-PK (KU60019, VE-821 and NU-7441, respectively) for 1 h before subjecting cells to 10 Gy of irradiation. Cell lysates were harvested 1 h after irradiation. The result is representative of two independent experiments. (**b**) ATM-deficient and -repleted cells were combined in the indicated percentages. Quantitations of pSMC1/SMC1 and pKAP1/KAP1 values were plotted against the ATM percentage. Results are based on data from four experiments.
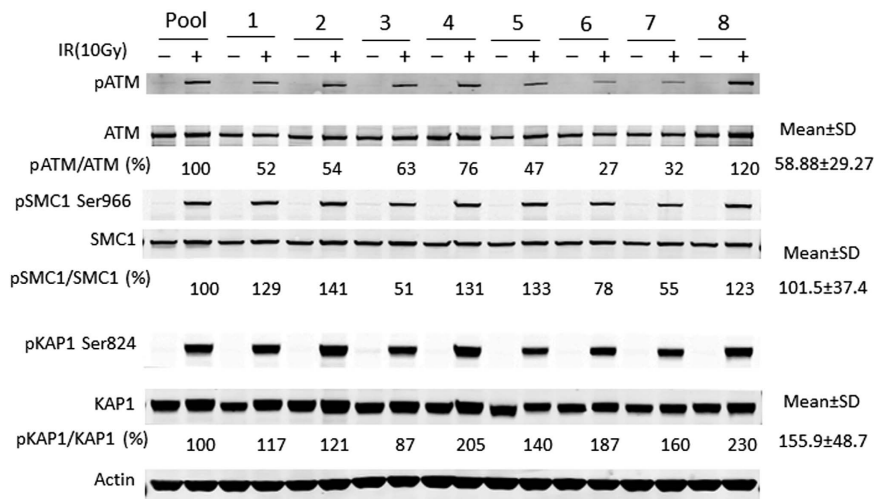


**Figure 2.** ATM function in pooled samples. Eight CLL samples from FISH normal patients were isolated and subjected to 10 Gy irradiation. The cell lysates were harvested 1 h after the treatment and immunoblotting was performed. The ratios of pATM/ATM, pSMC1/SMC1 and pKAP1/KAP1 of the Pool lysates were set as 100% and compared with those of the eight individual samples.

subjected to immunoblotting. When compared with the phospho/total values of the Pool, variations in the levels of phosphorylation of SMC1 and KAP1 were evident among the eight individual samples, indicating the heterogeneity within the CLL samples from different patients (Figure 2). We conclude that single samples are not representative of the population. Therefore, we combined lysates (Pool) for use as a positive comparator for the subsequent analyses of CLL samples that exhibit 11q deletion.

We applied this assay of ATM function to 46 archived or fresh CLL samples with 11q deletion to determine the function of the residual *ATM* allele. All samples were subjected to the identical treatment and evaluation as described above. The phosphorylation levels of the KAP1 and SMC1 proteins among the CLL patient

samples were calculated and compared with those of the Pool comparator. The ratios of phosphorylated to total SMC1 and KAP1 from 46 CLL samples were analyzed by the normal mixture model-based clustering method.[14] This determined that the cutoff numbers individually for both SMC1 and KAP1 to define ATM function deficiency were 21% and 55%, respectively, relative to the Pool value (Supplementary Figures 3A and B). Then, the normal mixture model-based clustering method was applied by considering the SMC1 proportion and KAP1 proportion simultaneously and a formula was generated to determine the ATM function (Figure 3a and Supplementary Figure 3C). Using this formula, we identified 13 ATM non-functional samples of 46 11q deletion CLL samples (Figure 3b). We evaluated the
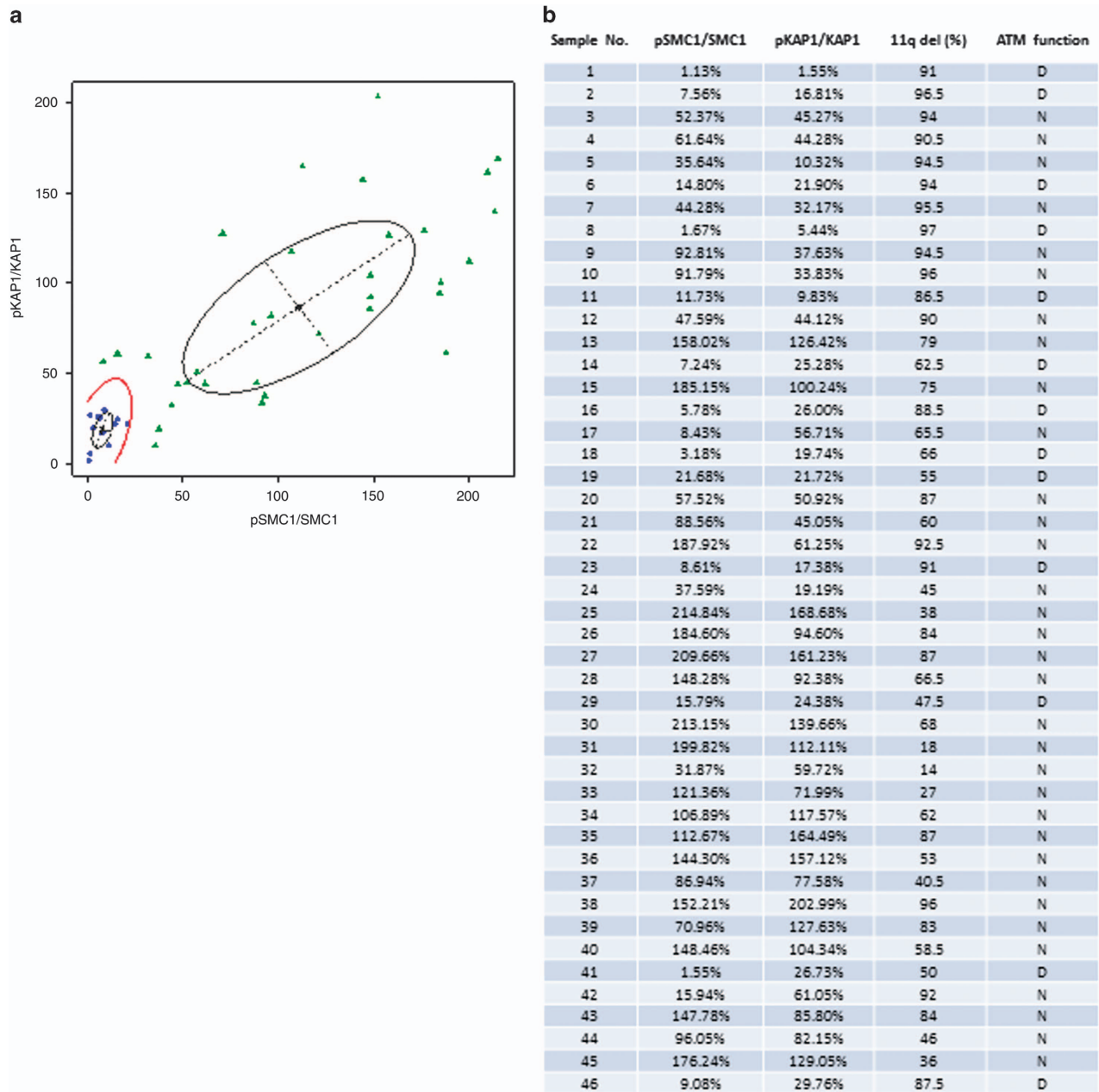
**a**



**b**

| Sample No. | pSMC1/SMC1 | pKAP1/KAP1 | 11q del (%) | ATM function |
|---|---|---|---|---|
| 1 | 1.13% | 1.55% | 91 | D |
| 2 | 7.56% | 16.81% | 96.5 | D |
| 3 | 52.37% | 45.27% | 94 | N |
| 4 | 61.64% | 44.28% | 90.5 | N |
| 5 | 35.64% | 10.32% | 94.5 | N |
| 6 | 14.80% | 21.90% | 94 | D |
| 7 | 44.28% | 32.17% | 95.5 | N |
| 8 | 1.67% | 5.44% | 97 | D |
| 9 | 92.81% | 37.63% | 94.5 | N |
| 10 | 91.79% | 33.83% | 96 | N |
| 11 | 11.73% | 9.83% | 86.5 | D |
| 12 | 47.59% | 44.12% | 90 | N |
| 13 | 158.02% | 126.42% | 79 | N |
| 14 | 7.24% | 25.28% | 62.5 | D |
| 15 | 185.15% | 100.24% | 75 | N |
| 16 | 5.78% | 26.00% | 88.5 | D |
| 17 | 8.43% | 56.71% | 65.5 | N |
| 18 | 3.18% | 19.74% | 66 | D |
| 19 | 21.68% | 21.72% | 55 | D |
| 20 | 57.52% | 50.92% | 87 | N |
| 21 | 88.56% | 45.05% | 60 | N |
| 22 | 187.92% | 61.25% | 92.5 | N |
| 23 | 8.61% | 17.38% | 91 | D |
| 24 | 37.59% | 19.19% | 45 | N |
| 25 | 214.84% | 168.68% | 38 | N |
| 26 | 184.60% | 94.60% | 84 | N |
| 27 | 209.66% | 161.23% | 87 | N |
| 28 | 148.28% | 92.38% | 66.5 | N |
| 29 | 15.79% | 24.38% | 47.5 | D |
| 30 | 213.15% | 139.66% | 68 | N |
| 31 | 199.82% | 112.11% | 18 | N |
| 32 | 31.87% | 59.72% | 14 | N |
| 33 | 121.36% | 71.99% | 27 | N |
| 34 | 106.89% | 117.57% | 62 | N |
| 35 | 112.67% | 164.49% | 87 | N |
| 36 | 144.30% | 157.12% | 53 | N |
| 37 | 86.94% | 77.58% | 40.5 | N |
| 38 | 152.21% | 202.99% | 96 | N |
| 39 | 70.96% | 127.63% | 83 | N |
| 40 | 148.46% | 104.34% | 58.5 | N |
| 41 | 1.55% | 26.73% | 50 | D |
| 42 | 15.94% | 61.05% | 92 | N |
| 43 | 147.78% | 85.80% | 84 | N |
| 44 | 96.05% | 82.15% | 46 | N |
| 45 | 176.24% | 129.05% | 36 | N |
| 46 | 9.08% | 29.76% | 87.5 | D |

**Figure 3.** ATM function in 46 11q deletion CLL samples. (**a**) The normal mixture density and boundary for clusters estimated based for phosphorylation SMC1 and KAP1 relative the Pool. (**b**) Analysis of pSMC1/SMC1 and pKAP1/KAP1 ratios of the 46 CLL samples. Quantitation of pSMC1/SMC1 and pKAP1/KAP1 was carried out by comparing the levels in these 46 samples against the levels of those in the Pool (set as 100%) statistical formula. D, deficient; N, normal.

phosphorylation of ATM as an indicator of ATM activation, and found that the ratio of pATM to ATM did not correlate with ATM function in these CLL samples ($P = 0.49$) (Supplementary Figure 4A). Although there was heterogeneity among samples of the proportion of cells that exhibited 11q deletion, the majority (37/46; 80%) of samples had the deletion in >50% of the cell population. There was no definitive association between percentage of 11q deletion and ATM function (Supplementary Figure 4B).

Targeted sequencing of *ATM* gene sequence in 11q deletion CLL samples

Mutations in the residual *ATM* allele are frequently found in 11q deletion CLL samples.[5] To determine whether there is a relationship between *ATM* gene mutation and the function of the ATM protein, we conducted targeted sequencing of the *ATM* gene in the 46 samples. Genomic DNA of T cells isolated and expanded from each of these samples was extracted to serve as a germline reference for the CLL cells, permitting comparisons of *ATM* sequence within individuals. Through a series of bioinformatics analyses, 27 *ATM* mutations were detected with a 10% variant allele frequency cutoff; the distribution of these mutations on the ATM protein is shown in Figure 4a. Among these mutations, 12 were somatic mutations. One mutation in a splicing site at exon 28 was found in two samples: a single sample (no. 11) was found to have two somatic mutations (Figure 4b). Fifteen other *ATM* mutations were recognized in this sequencing as
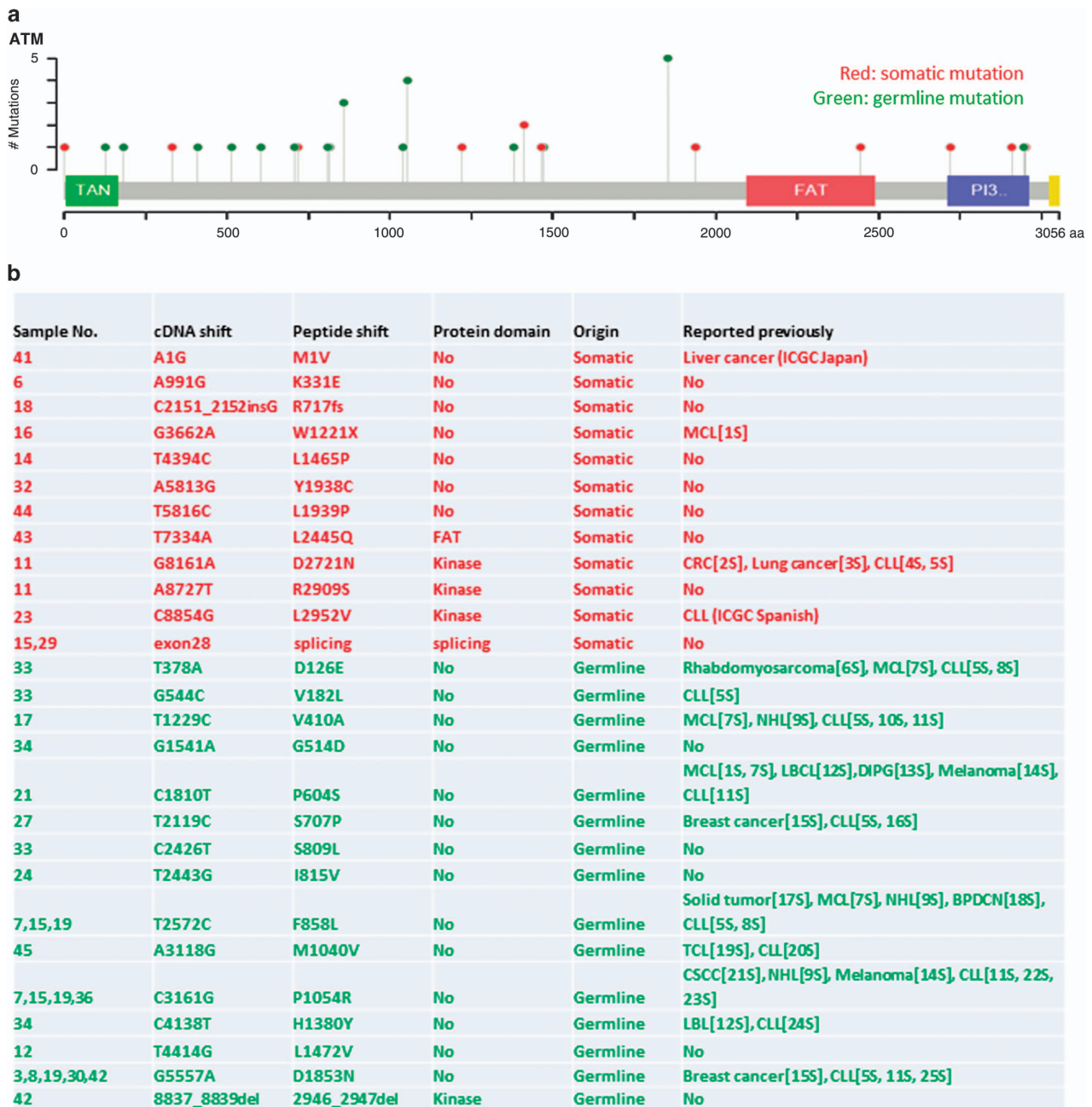
**a**



**b**

| Sample No. | cDNA shift | Peptide shift | Protein domain | Origin | Reported previously |
|---|---|---|---|---|---|
| 41 | A1G | M1V | No | Somatic | Liver cancer (ICGC Japan) |
| 6 | A991G | K331E | No | Somatic | No |
| 18 | C2151_2152insG | R717fs | No | Somatic | No |
| 16 | G3662A | W1221X | No | Somatic | MCL[1S] |
| 14 | T4394C | L1465P | No | Somatic | No |
| 32 | A5813G | Y1938C | No | Somatic | No |
| 44 | T5816C | L1939P | No | Somatic | No |
| 43 | T7334A | L2445Q | FAT | Somatic | No |
| 11 | G8161A | D2721N | Kinase | Somatic | CRC[2S], Lung cancer[3S], CLL[4S, 5S] |
| 11 | A8727T | R2909S | Kinase | Somatic | No |
| 23 | C8854G | L2952V | Kinase | Somatic | CLL (ICGC Spanish) |
| 15,29 | exon28 | splicing | splicing | Somatic | No |
| 33 | T378A | D126E | No | Germline | Rhabdomyosarcoma[6S], MCL[7S], CLL[5S, 8S] |
| 33 | G544C | V182L | No | Germline | CLL[5S] |
| 17 | T1229C | V410A | No | Germline | MCL[7S], NHL[9S], CLL[5S, 10S, 11S] |
| 34 | G1541A | G514D | No | Germline | No |
| 21 | C1810T | P604S | No | Germline | MCL[1S, 7S], LBCL[12S], DIPG[13S], Melanoma[14S], CLL[11S] |
| 27 | T2119C | S707P | No | Germline | Breast cancer[15S], CLL[5S, 16S] |
| 33 | C2426T | S809L | No | Germline | No |
| 24 | T2443G | I815V | No | Germline | No |
| 7,15,19 | T2572C | F858L | No | Germline | Solid tumor[17S], MCL[7S], NHL[9S], BPDCN[18S], CLL[5S, 8S] |
| 45 | A3118G | M1040V | No | Germline | TCL[19S], CLL[20S] |
| 7,15,19,36 | C3161G | P1054R | No | Germline | CSCC[21S], NHL[9S], Melanoma[14S], CLL[11S, 22S, 23S] |
| 34 | C4138T | H1380Y | No | Germline | LBL[12S], CLL[24S] |
| 12 | T4414G | L1472V | No | Germline | No |
| 3,8,19,30,42 | G5557A | D1853N | No | Germline | Breast cancer[15S], CLL[5S, 11S, 25S] |
| 42 | 8837_8839del | 2946_2947del | Kinase | Germline | No |

**Figure 4.** *ATM* mutations in 46 11q deletion CLL samples (>10% prevalence). (**a**) The mutation location map in the ATM protein. Green dots: germline mutations; red dots: somatic mutations. (**b**) Distribution of *ATM* mutations in 46 CLL samples. BPDCN, blastic plasmacytoid dendritic cell neoplasm; CLL, chronic lymphocytic leukemia; CRC, colon and rectal cancer; CSCC, cutaneous squamous cell carcinoma; LBCL, large B-cell lymphoma; MCL, Mantle cell lymphoma; NHL, non-Hodgkin lymphoma; TCL, T-cell leukemia. Reference listed are in the Supplementary Materials.

germline mutations. In the 12 CLL samples that harbor somatic mutations, 8 were deficient in ATM function. Only one of the 15 CLL samples (7%) with only germline mutations had diminished the ATM function, indicating that the germline mutations have minimal impact on ATM activity. Seventeen of the 26 samples with mutations retained ATM function. However, 4 of the 20 CLL samples in which no mutations were detected by targeted sequencing were determined to be non-functional (Figure 5a). Based on the sequencing results, no strong correlation was observed between the presence or absence of mutations in the

residual *ATM* gene of CLL cells with deletion 11q and ATM function (*P* = 0.34) (Figure 5b).

In considering a cause for the lack of function in the four samples that lacked mutations, we observed that all four possessed < 7% of Pool values of ATM protein levels (Table 1). Analysis of methylation patterns of the *ATM* gene was conducted to determine whether epigenetic silencing could account for low expression of ATM in these samples (Table 1). However, no evidence of excessive methylation was found in the ATM promoter region or intron–exon boundary for any of the samples.

RNA was not available from these samples to quantitate ATM transcript expression analysis by reverse transcription-polymerase chain reaction or for quantitation of noncoding RNA that could affect ATM expression.[22] Accordingly, no mechanism was established for the diminished ATM protein in samples that did not have mutations.

The progression-free survival was examined in 41 of the 11q deletion samples in which data were available. Patients with deficient ATM function had lesser progression-free survival (23 months) as compared with those with proficient ATM function (44 months) ($P = 0.48$; Supplementary Figure 4C), which is consistent with a previous report.[5] Interestingly, the median progression-free survival (44 months) for ATM-mutated samples was greater than ATM-unmutated samples (23 months), but the difference was not significant ($P = 0.93$; Supplementary Figure 4D). Future investigations of samples from homogeneously treated patients may provide a definitive answer to effect of loss of ATM function on clinical response.
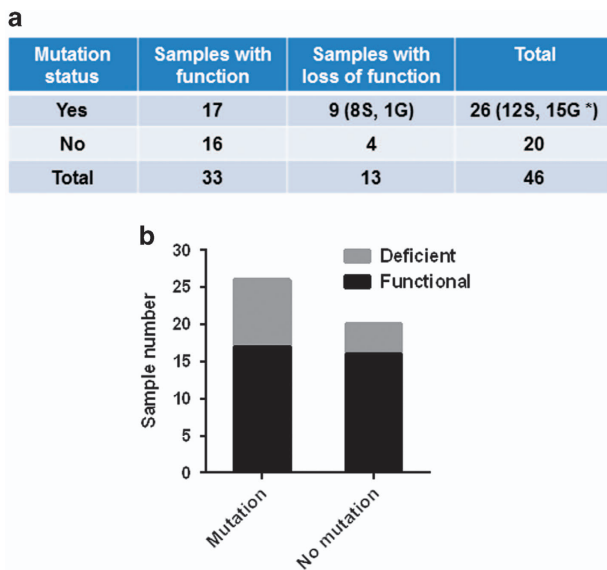


Figure 5. Relationship between ATM mutation and function. (a) ATM mutation and function distribution in all 46 CLL samples. G, germline mutation; S, somatic mutation. *One sample (no. 15) has both somatic and germline mutation. (b) Relationship between ATM mutation and function. P-value was calculated by Fisher's exact test.

## DISCUSSION

Current research suggests that deficiency of ATM function in malignancies may serve to guide evaluation of therapeutic strategies, enlighten disease prognosis and possibly contribute to clinical trial design. The lack of a consensus on a validated indicator of ATM function in clinical samples stimulated the present investigation. SMC1 and KAP1 were confirmed as unique substrates of ATM; their phosphorylation levels after irradiation were linearly correlated with ATM activity using A–T-mutant and -repleted cell lines. The ratios of phosphoprotein to total protein of SMC1 and KAP1 were used as indicators of ATM function in response to IR treatment. Using a Pool of eight FISH-negative CLL samples as a positive standard, this assay was applied in 46 primary CLL samples exhibiting deletion of the 11q region. Thirteen ATM function-deficient individuals were identified using a statistical model. Targeted sequencing of the entire coding region of the ATM gene on all 46 samples revealed 12 somatic and 15 germline mutations through bioinformatic analysis. However, this analysis failed to reveal a strong correlation between ATM function and mutation in the residual allele.

DNA methylation of the promoter region has been recognized as an alternative mechanism for gene inactivation in addition to deletions and mutations. Despite that aberrant methylation of the ATM promoter has been reported in various cancer types such as breast cancer,[23] head and neck cancer,[24] non-small-cell lung cancer[25] and in primary gastric lymphoma,[26] one study in CLL showed no apparent methylation was detected in ATM promoter region.[27] In concordance with this study, our methylation analysis in four CLL samples, which lost ATM function in the absence of a mutation, did not detect any promoter hypermethylation. ATM protein levels in all 46 samples were examined and no evident relationship between the protein and function was found. Unfortunately, RNA was unavailable from these samples to quantitate ATM transcript or noncoding RNA expression levels. The possibility of noncoding RNA regulation of ATM expression is supported by the report that miR-18a expression attenuated cellular repair of DNA DSBs by directly suppressing ATM in colorectal cancer and breast cancer.[28,29] Another study demonstrated that miR-421 suppressed ATM expression by targeting the 3'-untranslated region of ATM transcripts, and its expression was regulated by N-Myc in neuroblastoma.[22] Post-translational modification of ATM could also have a role in regulating ATM kinase activity. ATM undergoes autophosphorylation and becomes activated in response to DNA damage.[30] Three phosphatases, including PP2A, PP5 and WIP1, have been implicated in the control of ATM activation.[31–33] In addition to phosphorylation, it has been reported that TIP60, an acetyltransferase, acetylates ATM

Table 1. ATM promoter methylation status in seven 11q deletion CLL samples without ATM mutation

| Sample no. | ATM promoter Average methylation (%) | ATM intron 1/alternative exon 1 Average methylation (%) | ATM protein level (using Pool as 100%) | ATM function |
|---|---|---|---|---|
| 13 | 1.12 | 0.78 | 113 | Normal |
| 22 | 0.92 | 0.64 | 87 | Normal |
| 25 | 1.42 | 0.65 | 342 | Normal |
| 1 | 1.16 | 0.52 | 0.3 | Deficient |
| 2 | 0.95 | 0.96 | 2 | Deficient |
| 8 | 0.91 | 0.76 | 0.4 | Deficient |
| 46 | 1.23 | 0.68 | 6 | Deficient |
| S/W (intermediate methylated) | 39.53 | 42.13 | | |
| SssI (highly methylated) | 87.57 | 91.70 | | |
| WGA (unmethylated) | 7.72 | 0.33 | | |

Abbreviations: ATM, ataxia-telangiectasia-mutated; CLL, chronic lymphocytic leukemia; SssI, SssI-treated; S/W, equimolar mixture of SssI-treated and WGA-amplified; WGA, whole genome amplification.

on Lys3016, and this post-translational modification is important for ATM activation.[34,35]

ATM is a master regulator in the DNA damage repair signaling pathways.[36] Loss of ATM activity leads to the rare autosomal recessive disorder ataxia-telangiectasia. Importantly, cancer susceptibility occurs at especially high incidence (10–15%) of lymphoproliferative disorders in childhood or early adulthood.[37] In addition to CLL, the region on chromosome 11 containing the ATM gene is frequently deleted in Mantle cell lymphoma and T-cell prolymphocytic leukemia.[38] Deletion of 11q has been recognized as a poor prognostic factor for CLL in progression-free survival and overall patient survival.[2] Several studies have been conducted to test the clinical significance of ATM mutations in different cohorts of CLL patients. In one study in which the mutation status of ATM, regardless of cytogenetic background, in a cohort of 155 CLL tumors was examined, showed that ATM mutations were correlated with impaired overall and treatment-free survival.[6] In a following study, the same group discovered that the combination of 11q deletion and ATM mutation was associated with shorter progression-free and overall survival after first-line therapy.[5] Further, it was found that ATM heterozygous mutations did not influence CLL initiation, but affected disease progression.[39] In contrast, biallelic inactivation of ATM was associated with reduced patient survival.[7] Another independent investigation showed that ATM mutations were associated with a shorter treatment-free interval.[40] Finally, two other groups concluded that ATM aberrations had minimal impact on both progression-free and overall survival and could not support a major prognostic role in CLL.[41,42]

Owing to the lack in concordance in the functional significance of ATM mutation, a functional assay may be useful to interpret clinical outcomes. Identification of ATM function-deficient individuals may provide prognostic indications for these subgroups to therapies that target redundant, cooperative DNA damage response pathways such as ATR, DNA-PK and PARP1 inhibition. For example, a recent study reported ATR inhibition led to synthetic lethality in ATM-defective CLL cells.[43] Another work showed that the PARP1 inhibitor, olaparib, selectively killed ATM-deficient lymphoid tumor cells.[44] Interestingly, one group found that ATM-defective cells displayed strong non-oncogene addiction to DNA-PKcs (DNA-dependent protein kinase catalytic subunit), and that these cells underwent apoptosis after exposure to DNA-PKcs inhibitors.[45]

Therefore, the assay described, in which of the phosphorylation of ATM-specific targets (SMC1 and KAP1) are collectively assessed, could provide a reliable way to determine ATM function in cancer patients and guide subsequent treatment options.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

Conception and design: YJ, XL and WP; provision of study materials or patients: WW and MJK; collection and assembly of data: YJ; data analysis and interpretation: YJ, HC, XS, PAT and WP; manuscript writing: YJ and WP; final review and approval of manuscript: all authors.

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