ATM gene alterations in chronic lymphocytic leukemia patients induce a distinct gene expression profile and predict disease progression

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
The genetic characterization of chronic lymphocytic leukemia cells correlates with the behavior, progression and response to treatment of the disease.

Design and Methods
Our aim was to investigate the role of *ATM* gene alterations, their biological consequences and their value in predicting disease progression. The *ATM* gene was analyzed by denaturing high performance liquid chromatography and multiplex ligation probe amplification in a series of patients at diagnosis. The results were correlated with immunoglobulin gene mutations, cytogenetic abnormalities, ZAP-70 and CD38 expression, *TP53* mutations, gene expression profile and treatment-free interval.

Results
Mutational screening of the *ATM* gene identified point mutations in 8/57 cases (14%). Multiplex ligation probe amplification analysis identified six patients with 11q deletion: all of them had at least 20% of deleted cells, analyzed by fluorescent *in situ* hybridization. Overall, *ATM* point mutations and deletions were detected in 14/57 (24.6%) cases at presentation, representing the most common unfavorable genetic anomalies in chronic lymphocytic leukemia, also in stage A patients. Patients with deleted or mutated *ATM* had a significantly shorter treatment-free interval compared to patients without *ATM* alterations. *ATM*-mutated cases had a peculiar gene expression profile characterized by the deregulation of genes involved in apoptosis and DNA repair. Finally, definition of the structure of the *ATM*-mutated protein led to a hypothesis that functional abnormalities are responsible for the unfavorable clinical course of patients carrying these point mutations.

Conclusions
ATM alterations are present at diagnosis in about 25% of individuals with chronic lymphocytic leukemia; these alterations are associated with a peculiar gene expression pattern and a shorter treatment-free interval.

Key words: *ATM*, chronic lymphocytic leukemia, gene expression profiling, MLPA, del11q.

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the western hemisphere. It is characterized by a clonal accumulation of small, mature-looking lymphocytes in the blood, bone marrow and secondary lymphoid tissues.[1] The disease has a highly variable clinical course, with some patients surviving for many years without requiring treatment and others who have a rapidly progressing disease, associated with a short life expectancy, despite aggressive treatment.

Several biological and genetic properties of the leukemic cells, such as the mutational status of the immunoglobulin heavy chain variable genes (*IGHV*),[2] chromosome aberrations,[3] CD38 and ZAP-70 expression,[4,5] and p53 dysfunction,[6] bear an important prognostic value and have allowed patients to be stratified into risk categories. These parameters are in fact important independent predictors of disease progression and survival.

The deletion of chromosome 11q22-q23, which occurs in 10-20% of cases,[3] represents the second most common genetic abnormality in CLL and defines a subgroup of patients with progressive disease and, overall, an unfavorable prognosis;[7] in fact, leukemic cells show increased survival rates, possibly because of inhibited apoptosis and alterations of the genes involved in cell-cycle control and cell survival.[8]

The *ATM* (ataxia-telangiectasia mutated) gene maps to chromosome 11q22-q23 within the minimal region of loss described in CLL[9] and several data indicate that 11q deletion results in *ATM* gene inactivation.[10] The *ATM* gene is a member of the phosphatidylinositol-3 kinase (PT3K) family of genes and consists of 66 exons, of which 62 are coding exons.[11] The ATM protein is a nuclear serine/threonine kinase of 350 kDa whose activities are induced by chromosomal double-strand breaks that arise endogenously or after exposure to DNA-damaging agents, including ionizing radiation and drugs.[12]

The ATM protein is a pleiotropic molecule that protects the integrity of the genome by regulating the cell-cycle arrest at G1/S and G2/M to prevent processing of damaged DNA, and activating DNA-repair pathways and inducing apoptosis if the DNA damage cannot be repaired.[13] Many of these effects are mediated via a phosphatidylinositol-3 kinase domain in the C-terminus of the ATM protein (residues 2656-3056). The homozygous mutation of the *ATM* gene is known to be the cause of ataxia-telangiectasia (A-T), an autosomal recessive disorder characterized by neurological and immunological symptoms, radiosensitivity and predisposition to cancer, particularly of the lymphoid system.[14] Several epidemiological studies suggest that the frequency of A-T heterozygous carriers ranges between 0.5% and 1% in different countries; these individuals have a significantly increased risk of developing breast cancer[15] and CLL.[16,17] One third of CLL patients have an inactive *ATM* and exhibit defects in the p53 damage response and in apoptosis induced by ionizing radiation.[18,19] These findings have considerable clinical implications because *ATM* mutations may be important in predicting potential treatment failure.[20]

In the present study we examined the mutational status of the *ATM* gene in a series of CLL patients studied at diagnosis. A multiplex gene dosage analysis of the *ATM* gene was also performed by multiplex ligation probe amplification (MLPA). The results were then correlated with the known biological prognostic factors. Modeling structural analysis of the mutated ATM protein was carried out in order to understand the effects of the mutation on the behavior of the neoplastic cells.

Design and Methods

Chronic lymphocytic leukemia patients

We analyzed samples from 57 untreated CLL patients, collected between 1997 and 2005 at the Hematology Institute of the "Sapienza" University of Rome. There were 28 females and 29 males with a median age of 50 years (range, 29-64). The diagnosis of CLL was based on the presence of more than 4,000 clonal lymphocytes/μL in the peripheral blood with a typical CLL immunophenotype (CD5/CD20$^+$, CD23$^+$, weak CD22$^+$, weak sIg$^+$, CD10$^-$) and morphology. According to the Binet staging system, 42 patients were in stage A, 12 in stage B and 3 in stage C. The patients had a median of 27,679 lymphocytes/L (range, 4,118-212,400) at the time of the study. The patients' characteristics are presented in *Online Supplementary Table S1*.

All samples were analyzed for CD38 and ZAP-70 expression, for *IGHV* status and for *TP53* mutations as previously described.[21]

This study was approved by the Institutional Review Board of the Department of Cellular Biotechnologies and Hematology, "Sapienza" University of Rome. All patients and controls gave their informed consent to blood collection and to the biological analyses included in the present study according to the Declaration of Helsinki.

DNA was extracted from the leukemic cells of the 57 unrelated patients and tumor DNA was analyzed to determine *ATM* mutations. The detected *ATM* alterations were investigated in DNA from patient-matched buccal cells to determine their germline or somatic nature.

Denaturing high performance liquid chromatography analysis of the ATM gene

Mutation scanning was performed by denaturing high performance liquid chromatography (DHPLC) analysis, following previously published protocols[22,23] in which a 86% mutation detection rate in *ATM*-mutated patients and a 100% specificity has been reported.

Sixty-two out of the 66 exons of *ATM*, along with exon-intron junctions, were amplified by polymerase chain reaction (PCR).[22] DHPLC analysis was performed as previously described.[22,23] All amplification products showing an abnormal elution profile were re-amplified and sequenced in forward and reverse directions using the BigDye Terminator chemistry and an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems). The pathogenic role of novel missense and intronic changes was evaluated by screening 360 control chromosomes from 180 unrelated healthy individuals.

Multiplex ligation probe amplification analysis of the ATM gene

To estimate the contribution of single and multi-exon *ATM* gene copy-number changes, which could be missed by large fluorescent *in situ* hybridization (FISH) probes, a multiplex ligation probe analysis (MLPA) was performed using the SALSA MLPA kit P123 *ATM,* available from MRC Holland (MRC-Holland, Amsterdam, The Netherlands).

This assay consists of two reaction mixes containing probes for 33 of the 66 constitutive *ATM* exons and control probes for sequences located in other genes. An aliquot of 150 ng of denatured genomic DNA was used in the overnight annealing of the exon-specific probes and subsequent ligation reaction. PCR was

performed with FAM-labeled primers using 10 mL of ligation reaction. The amplification products were separated and quantified using an ABI Prism 3130 Genetic Analyzer (Applied Biosystem). The peak area for each fragment was measured with GeneScan Analysis software V.3.7 (Applied Biosystems) and the data were analyzed with the Coffalyser software (MRC-Holland). The results are reported as the ratio between allele copy numbers (relative copy number) of the cells from a CLL patient and healthy controls. A ratio of 1 should be obtained if both alleles are present; a reduction or an increase in the peak area values to 0.7 or 1.3 was considered an indication of a deletion or a duplication, respectively.

Statistical analysis of treatment-free interval

The treatment-free interval was calculated from the date of diagnosis to first treatment. The probability of not requiring treatment was estimated using the Kaplan-Meier test; since no patient died before treatment, it was not necessary to estimate treatment-free interval by means of cumulative incidence curves, considering death before treatment as a competing risk. The log-rank test was used to test differences between groups.

RNA extraction and oligonucleotide microarray

Total RNA was extracted using the RNeasy mini procedure (Qiagen), according to the manufacturer's instructions. All samples analyzed contained at least 90% leukemic cells. HGU133 Plus 2.0 gene chips (Affymetrix, Santa Clara, CA, USA) were used to determine gene expression profiles. Further details are provided in the *Online Supplementary Design and Methods*.

Statistical methods for microarray analysis

Oligonucleotide microarray analysis was performed with dChip software (*www.dchip.org*) Model based expressions were computed for each array and probe set using the PM-MM model.[24] Unsupervised clustering was performed as described by Eisen *et al.*[25] Further details are provided in the *Online Supplementary Design and Methods*.

A t-test was applied to identify genes differentially expressed between different CLL subclasses: probe-sets were required to have an average expression of greater than 100 in at least one group, a *P* value of 0.05 or less and a fold change of 1.5 or more. Gene functional annotations were identified using the DAVID database (*http://david.abcc.ncifcrf.gov*).

Real-time quantitative polymerase chain reaction analysis

One microgram of total RNA was reverse transcribed using the Advantage RT-for-PCR Kit (Clontech, Mountain View, CA, USA). Real-time quantitative-PCR (Q-PCR) analysis was performed with an ABI PRISM 7500 sequence detection system and SYBR green dye (Applied Biosystems). Primers were designed using Primer Express 1.5.1 software (Applied Biosystems).

Further details are provided in the *Online Supplementary Design and Methods*; gene symbols and primers are listed in *Online Supplementary Table S2*.

Molecular modeling of the ATM kinase domain

The structure of the PI3K-like domain of ATM in the amino acid interval 2623-2953 was built by homology modeling using the program MODELLER (release 9v3)[26] and using as a template the structure of the homologous porcine PI3Kγ in complex with ATP (Protein Data Bank, PDB, entry 1E8X), according to alignment of sequence and secondary structure elements (the latter are predicted by PSIPRED for ATM and experimental for porcine PI3Kγ), as shown in *Online Supplementary Figure S1*.

The alignment allowed identification of the nucleotide binding loop in the N-terminal side of the kinase domain of ATM at about amino acids 2694-2699, because of the congruence with the typical secondary structure features for this region of the protein. Amino acid interval 2795-2830 of ATM emerges as an insertion with respect to the porcine PI3Kγ sequence and has not been modeled. However, this part of the protein does not appear to contribute to the kinase fold because it shows a less strict conservation of amino acids; in addition, the presence of several charged residues suggest solvent exposure with probable implications in the mechanisms of ATM activation and/or substrate recognition. The ATP co-factor has been modeled on the kinase domain of ATM according to the binding conformation of the ATP ligand reported in the crystallographic structure of PI3Kγ.

To assess the congruence of the proposed architectural model, we also verified whether amino acids crucial for kinase activity are properly located inside the structure. Specifically, we identified the position of the lysine that interacts with the phosphate group of ATP and the aspartic acid that acts as proton acceptor, which are the two active site residues directly involved in kinase activity. The former of these two residues in ATM appears to be the invariant Lys2717, because it aligns accurately with Lys833 of porcine PI3Kγ, which in turn is known as the active site lysine for this homologous kinase.[27] The proton acceptor residue in ATM turns out to be the invariant Asp2870 given its geometric coincidence with the annotated catalytic aspartic acid residue of another structurally characterized kinase (PDB structure 1VYW, cell division protein kinase 2) that is observed after rigid superposition of this latter structure with our model.

Results

Denaturing high performance liquid chromatography analysis for ATM mutations

Fifty seven CLL patients were screened for mutations in the 62 coding exons of the *ATM* gene. Mutational screening of the *ATM* gene identified eight (14%) patients with heterozygous mutations: one frameshift 2502insA, one splicing mutation IVS29+5G>A, and six missense mutations, 8095C>T (P2699S), 8071C>T (R2691C), 2476A>C (I826L) and 1435G>T (D479Y) in three patients: given the relatively high incidence of the last mutation, in order to exclude the possibility of contamination, screening for the presence of this mutation was performed, and its presence was confirmed in two different DNA aliquots from the same individual (Table 1). In 4/8 cases, the *ATM* mutations were also looked for in a non-neoplastic cell population, namely buccal cells, to verify whether the alteration was germline or carried only by the neoplastic cells: in one of four cases the mutation was germline (Table 1).

In addition, nine different variants or polymorphisms, defined on the basis of referenced data, were found in 14 patients (*Online Supplementary Table S3*); their functional significance is unknown. *ATM* mutations, variants and polymorphisms were also evaluated in 180 healthy volunteers, to test, in matched controls, whether these variants segregate in the Italian population and to determine their frequency (*Online Supplementary Table S3*).

Multiplex ligation probe amplification analysis for ATM deletions/duplications

All 57 CLL patients were analyzed for *ATM* gene copy number variations by MLPA. This method identified an entire gene deletion in six patients. In all six samples, MLPA

analysis showed a significant decrease in the peak heights for all *ATM* exons with a mean relative copy number of 0.58. This finding confirmed previous results obtained by FISH analysis, showing a deletion in at least 20% of the leukemic cells. No deletion was found in patients carrying point mutations.

Relationship between ATM gene mutations and prognostic factors

Patients with ATM point mutations

Analysis of the sequence of *IGHV* genes in the eight *ATM*-mutated cases showed that six had unmutated *IGHV* and two (MR 3664; AE 5646) had mutated *IGHV* (Table 2).

ZAP-70 was expressed in four out of eight *ATM*-mutated cases (MR 3664; PD 3988; VA 4046; IA 5948). The CD38 antigen was present in more than 7% of leukemic cells in five out of eight cases (CF 5116; ID 5637; PD 3988; VA 4046; IA 5948), but only in one (VA 4046) were more than 20% of the cells positive. Several cytogenetic imbalances, evaluated by FISH, were found in *ATM*-mutated patients: deletion 13q14 in 5/8 patients (ID 5637; MR 3664; PD 3988; AE 5646; CF5116), deletion 14q32 in 2/8 patients (CF 5116; MR 3664) and deletion 17p13 in three of the eight patients, but in only one case (PD 3988) were more than 20% of the cells positive. Two of eight *ATM*-mutated cases had a coexisting mutation in the *TP53* gene (PD 3988; IA 5948). Deletion 11q23 was negative in all *ATM*-mutated patients, but patient CF 5116 developed the deletion in 45% of leukemic cells at the time of disease progression.

Six patients were in stage A and two in stage B (GF 3706;PD 3988); three patients (AE 5646; GF 3706; PD 3988) had lymphadenopathy. At the time of data analysis, six of eight patients with *ATM* mutation had undergone treatment (MR 3664; PD 3988; GF 3706; ID 5637; CG 5116) and the median treatment-free interval was 30.0 months.

Patients with ATM deletions

All cases showing a significant reduction of *ATM* gene expression, evaluated by MLPA analysis, had a proportion of 11q23 deleted cells greater than 20% (Table 2). One case had a concomitant 17p13 deletion in 7% of the leukemic cells.

All six patients had unmutated *IGHV*. CD38 was positive in four of the six cases (CS 5700; PF 5216; PA 5704; VR 3835) and in all more than 20% of the leukemic cells expressed the antigen. ZAP-70 was positive in all cases.

Five patients were in stage A and one in stage B (CC 5394); four patients (CC 5394; CS 5700; PF 5216 VR 4046) had lymphadenopathy. At the time of data analysis, all 11q23 deleted patients had been treated and the median treatment-free interval was 23.5 months.

Patients without ATM mutations or deletions

Forty-three of the 57 CLL cases analyzed showed no *ATM* gene mutation or 11q23 deletion (Table 2). Two patients had del17p13, but only one in more than 20% of the leukemic cells, and one patient had a *TP53* gene mutation. In 16/43 (37%) cases, unmutated *IGHV* gene status was recorded. CD38 was positive in 8/43 (19%) cases and ZAP-70 was expressed in 12/39 (31%) patients. Thirty-one patients were in stage A, nine in stage B and three in stage C. At the time of data analysis, 26/43 patients had been treated and the median treatment-free interval was 64.2 months. When *ATM*-mutated and deleted patients were compared to patients without *ATM* alterations, the difference in treatment-free interval was statistically significant (P=0.0032) (Figure 1).

Microarray analysis in chronic lymphocytic leukemia cells with ATM point mutations

To evaluate the effects of *ATM* mutations on CLL cells, we performed a gene expression profile analysis on 41 of the 57 CLL patients with known *ATM* mutational status. We first utilized an unsupervised approach applying non-specific filtering criteria: hierarchical clustering based on a list of 226 selected genes showed that three of five *ATM*-mutated cases were included in the same cluster of patients; of note, two samples harbored the same *ATM* mutation (1435G>T) (*data not shown*).

Subsequently, we performed a supervised analysis comparing the *ATM*-mutated cases with the remaining CLL samples; as shown in Figure 2A, this approach revealed a common pattern of expression for CLL cases with *ATM* mutations, identifying a set of 32 differentially expressed genes. Among these, we found several genes involved in signal transduction (*TGFBR3*, *AXIN2*, *CD180*, *GABRB2*, *BACE2*), regulation of transcription (*RXRA*, *EIF4A*, *XBP1*),

Table 1. *ATM* gene point mutations in CLL patients.

ID N.	Patients	*ATM* gene mutation					
		Nucleotide Change	Amino acid Change	Type	Exon/ Intron	Germline (G)/ Somatic (S)	Allelic Status
3664	M.R.	1435G>T	D479Y	Missense	12	n.e.	Heterozygous
5948	I.A.	1435G>T	D479Y	Missense	12	S	Heterozygous
5646	A.E.	1435G>T	D479Y	Missense	12	n.e.	Heterozygous
3988	P.D.	2476>C	I826L	Missense	19	n.e.	Heterozygous
4046	V.A.	2502insA	–	Frameshift	19	n.e.	Heterozygous
5116	C.F.	IVS29+5G>A	–	Splicing	29	S	Heterozygous
3706	G.F.	8095C>T	P2699S	Missense	57	S	Heterozygous
5637	I.D.	8071C>T	R2691C	Missense	57	G	Heterozygous

n.e.: not evaluated.

Table 2. Biological and clinical features of the CLL patients studied.

Biological features	Patients with *ATM* mutations (n=8)	MLPA+ (n=6)	Patients without *ATM* gene alterations (n = 43)
del11q22.3 (FISH)			
>5% <20%	0/8	0/6	0/43
>20%	0/8	6/6 (100%)	0/43
del17p13.1 (FISH)			
>5% <20%	2/8 (25%)	1/6 (17%)	1/43 (2%)
>20%	1/8 (13%)	0/6	1/43 (2%)
IGHV			
unmutated	6/8 (75%)	6/6 (100%)	16/43 (37%)
mutated	2/8 (25%)	0/6	27/43 (63%)
ZAP-70>20%	4/8 (50%)	6/6 (100%)	12/39 (31%)
CD38>7%	5/8 (62%)	4/6 (67%)	8/43 (19%)
TP53 mutated	2/8 (25%)	0/6	1/43 (2%)
N. of treated patients	5/8 (62.5%)	6/6 (100%)	26/43 (60.4%)
TFI median (months)	30.0	23.5	64.2

TFI: treatment-free interval.

angiogenesis (*LAMA5*, *COL4A3*, *TMPRSS6*), apoptosis and cell-cycle regulation (*SRGN*, *LY86*, *SEPT10*) (*Online Supplementary Table S4*). Remarkably, similar results were obtained when the same comparison was performed excluding MLPA-positive cases (*data not shown*): this approach was undertaken to prove that the signature of *ATM* mutations is independent of 11q23 deletions.

Furthermore, given the documented association between *ATM* mutations and unmutated *IGHV* genes,[20] we compared *ATM*-mutated *versus ATM* wild-type cases exclusively in CLL with unmutated *IGHV*. This analysis provided even more interesting results, as shown by a more homogeneous pattern of expression and the identification of a larger set of differentially expressed genes (Figure 2B).

Microarray analysis in chronic lymphocytic leukemia cells with ATM deletions

The unsupervised analysis on CLL samples highlighted that four of six MLPA-positive patients were included in the cluster with *ATM*-mutated samples mentioned above (*data not shown*).

We subsequently performed a supervised analysis using a t-test between MLPA-positive cases and the other CLL samples, independently of *ATM* mutations (Figure 3A). This comparison identified 98 differentially expressed genes, as reported in the *Online Supplementary Table S5*. Among the more significant functional groups, we found different genes involved in signal transduction (*TCL1A*, *P2RX1*, *CNR1*, *IL10RA*, *CXCR5*, *CACNA1A*, *FMOD*, *TXNDC5*), regulation of transcription (*RXRA*, *BMI1*, *ZNF92*, *NR4A2*, *EIF3C*, *HOXC4*, *ZNF331*), cell adhesion (*PCDH9*, *SIGLEC10*, *VCL*, *LY9*, *COL18A1*, *CNTNAP2*), lipid metabolism (*APOD*, *ALG13*, *NPC2*, *FDX1*, *ALOX5*, *TSPO*, *PAFAH1B2*, *NRIP1*) and cytoskeleton organization (*DMD*, *ADD3*, *TUBB6*).

Moreover, our results highlighted a more distinctive signature associated with *ATM* deletions, coupled with a concomitant gene dosage effect. In fact, among the down-modulated genes, we detected reduced expression of several transcripts localized on the chromosome region 11q22-q23, such as *ATM*, *FDX1*, *MLL*, *CUL5*, *IL10RA*, *BIRC3*, *CXCR5*, *UBE4A*, *TMEM123*, *CCDC84*, *PAFAH1B2*, *CWF19L2* and *KIAA0999* genes.

In line with these findings, the decrease of expression levels of this set of genes correlated with the percentage of cells carrying the deletion (Figure 3B).

Furthermore, as already done for *ATM* mutations, in order to exclude the effects of *IGHV* mutational status, the same analysis was performed exclusively on CLL samples with unmutated *IGHV*, achieving analogous results (*data not shown*).

Validation of gene expression data by quantitative polymerase chain reaction analysis

To validate the microarray results, we performed a Q-PCR analysis on five CLL patients with *ATM* mutations, five MLPA-positive cases and five CLL without *ATM* alterations. As expected, Pearson's correlation index between the gene expression and Q-PCR ΔC_T values was high, confirming a good concordance of results from these two techniques.

Among the transcripts differentially expressed in the *ATM*-mutated *versus ATM* WT CLL selected by microarray, Q-PCR confirmed the significant up-regulation of *TGFBR3* (P=0.034) and *XBP1* (P=0.045) and significant down-modulation of *SEPT10* (P=0.05) in the former subgroup of patients (*Online Supplementary Figure 2A*). Similarly, Q-PCR analysis showed significantly different levels of expression

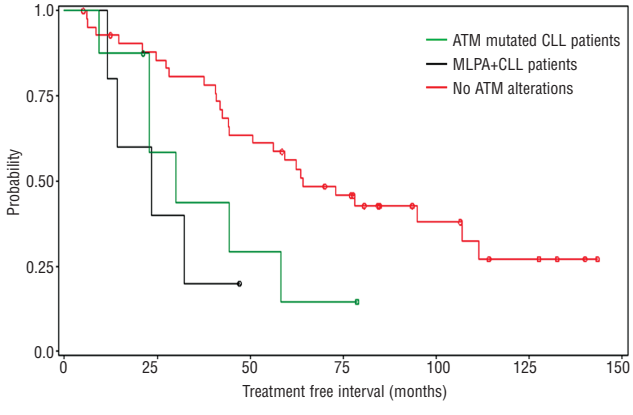

Figure 1. Statistical analysis of treatment-free survival. Evaluation of treatment-free interval in *ATM*-mutated and deleted CLL patients compared to patients without *ATM* alterations.

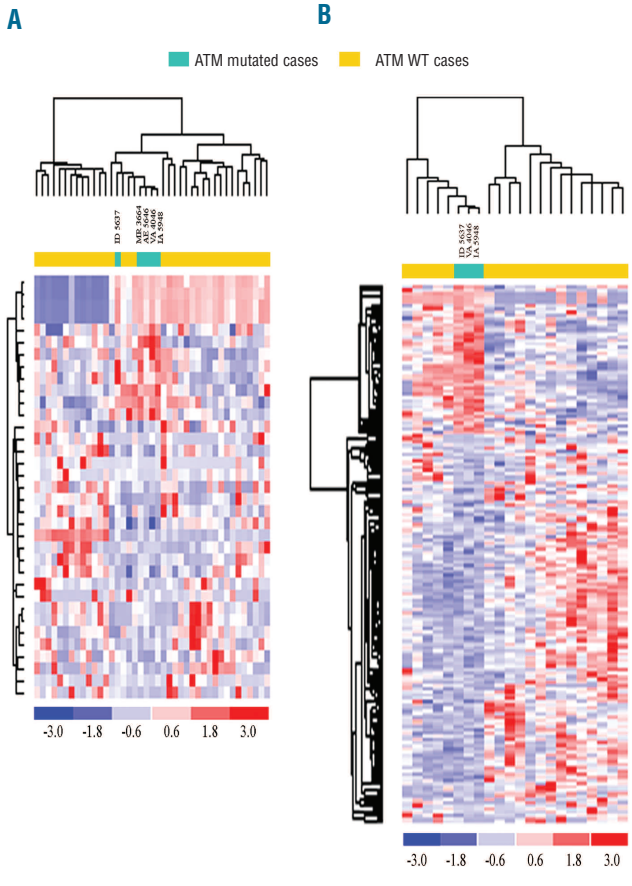

Figure 2. Comparison between *ATM*-mutated and non-mutated CLL patients. Differentially expressed genes between *ATM*-mutated and *ATM* wild-type (WT) cases in all the CLL patients analyzed (A) and in *IGHV* unmutated subjects (B). Upper legend: green represents *ATM*-mutated cases, yellow *ATM* WT cases. Relative levels of gene expression are depicted with a color scale: red represents the highest level of expression and blue represents the lowest level.

of *ATM* (*P*=0.039), *BIRC3* (*P*=0.0060), *TCL1A* (*P*=0.0024) and *TSPO* (*P*= 0.0014) between MLPA-positive and MLPA-negative cases (*Online Supplementary Figure 2B*).

Furthermore, we also evaluated the expression of a set of transcripts commonly deregulated in CLL with *ATM* alterations. In agreement with the gene expression data, *BACE2* and *TMPRSS6* were significantly down-regulated in both *ATM*-mutated and deleted patients, whereas *PCDH9* and *RXRA* were modulated in the opposite way in these two subclasses compared to the other CLL (*Online Supplementary Figure 2C*).

Finally, when we extended the analysis to an additional cohort of cases, including five CLL with *ATM* point mutations and five CLL with del11q, comparable results were obtained (*data not shown*).

Modeling of ATM protein mutations

Mutation D479Y was analyzed since it was detected in three *ATM*-mutated cases (Table 1). The understanding of the implications of this amino acid change on ATM function was difficult, since this region of the protein has so far not been studied. D479Y is included in the α-helix formed by amino acids 478-494 (secondary structure prediction by PSIPRED) and is highly conserved across species, having only glutamic acid, another negatively charged residue, as a much less frequent alternative. These features suggest the importance of this residue.

To understand the effects of R2691C and P2699S mutations we built the structure of the PI3K-like domain of ATM by homology modeling. The match between the pattern of secondary structures of ATM kinase and PI3Kγ (*Online Supplementary Figure S1*) allowed an unambiguous localization of the sites of R2691C and P2699S mutations in the pocket that binds the ATP co-factor (Figure 4). The R2691C mutation implies replacement of a large, positively charged arginine by a small, neutral cysteine residue, introducing significant structural and electrostatic changes in the ATP binding pocket. As for the P2699S mutation, according to the alignment and predicted secondary structure, the presence of a proline at position 2699 suggests that this residue acts as a breaker of the β-sheet formed by amino acids 2700-2706 (proline residues are commonly found as α-helix and β-sheet disruptors), thus initiating the formation of a reverse turn that is followed N-terminally by another β-sheet (amino acid 2690-2693). Such a secondary structure arrangement is essential for kinases and it is likely to be lost in cases with the P2699S mutation in which the invariant proline is replaced by a serine.

Given such important effects in a region critical for the binding of the co-factor, R2691C and P2699S mutations are

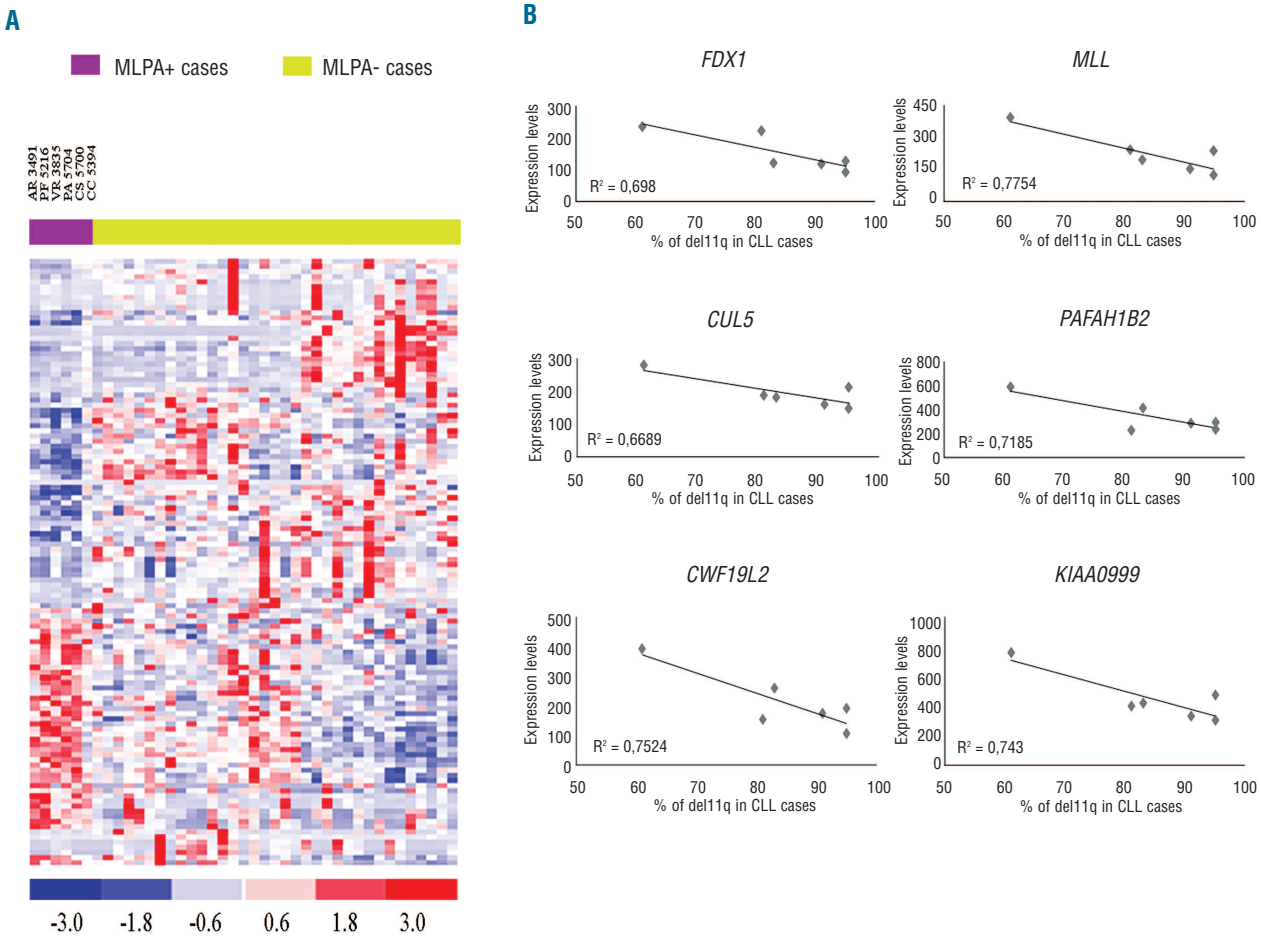

Figure 3. Comparison between MLPA+ and MLPA- CLL patients. Identification of 98 differentially expressed genes between MLPA+ cases and the remaining CLL samples. Upper legend: purple represents MLPA+ cases, light green MLPA- cases (A). Correlation between percentage of 11q22-23 deleted cells and expression levels of 6/13 transcripts localized on this chromosome region (B).

each expected to impair the kinase activity of ATM.

I826L modeling was not evaluated since the mutation falls outside the PI3K-like domain.

Discussion

In this study we analyzed the *ATM* gene in CLL patients, since 11q22-q23 deletion, in which the gene is located, represents the second most common cytogenetic imbalance and a biological parameter associated with an unfavorable prognosis.[3,7] We investigated whether CLL cells that carry *ATM* gene mutations and/or deletions showed a peculiar behavior, whether there was a molecular explanation and whether a particular type of therapy should be administered.[28] *ATM* gene mutations without 11q22.23 deletion were observed in 8/57 patients, indicating that this gene is often (14%) affected in CLL. Notably, all the patients were evaluated at diagnosis and before any treatment. The few reported data concerning the frequency of *ATM* gene mutations in untreated CLL patients are in agreement with our results (12%).[20] No other data are available concerning Italian patients with CLL. All point mutations but one (2502insA)[29] detected in this study are reported for the first time in CLL patients.

Our results suggest that the *ATM* gene behaves like the *TP53* gene:[19] deletions and mutations can be independent processes, but both affect prognosis. Considering both deletions and mutations of *ATM*, these alterations are present in a highly significant proportion of CLL patients at presentation (24.6%). Notably, in this study only patients 65 years or below were investigated and this could account for the frequency of the mutations.[30,31]

The majority of patients with *ATM* mutations showed poor prognostic biological features, i.e. unmutated *IGHV*, and ZAP-70 and CD38 expression,[32] More importantly, the majority of *ATM* mutated patients required treatment for disease progression over a short observation period: 100% of *ATM*-deleted patients and 62.5% of patients with *ATM* point mutations needed treatment within a median of 23.5 and 30.0 months after diagnosis, respectively. Overall, the treatment-free interval of CLL patients with *ATM* alterations was significantly shorter than that of patients without such abnormalities (64.2 months). These findings extend previously published data[7,20,30] In an attempt to explain this phenomenon, we measured the functional consequences of *ATM* deletions and point mutations by evaluating gene expression profiles. By supervised analysis, leukemic cells carrying the 11q22.23 deletion (cut-off >20%), showed down-modulation of the *ATM*, *MLL*, *CUL5* and *BIRC3* genes involved in the apoptosis machinery and DNA repair, and mapping to the 11q23 region, thus pointing to a gene dosage effect.[33,34] *ATM* down-modulation, also validated by Q-PCR analysis, represents a *bona fide* result.

It has been recently reported that other genes (i.e. *NCAM1*, *TTC12*, *ANKK1*, *DRD2*, *TMPRSS5*, *ZW10*, *USP28*, *HTR3B*, *HTR3A*, *PLZF*, *NNMT*, *C11orf71*, *RBM7*, *REXO2*, *FAM55A*, *FAM55B* and *TSLC1*) are included in the minimally deleted region on 11q;[35] in our cohort, these transcripts were down-modulated, but without significant differences when compared to the entire CLL series.

Similarly, Ouillette *et al.*[36] identified a frequent association between *ATM* deletions and monoallelic loss of *Mre11* and/or *H2AFX*; in line with these findings, mRNA levels were lower, but not significantly so, in cases with del11q compared with the other CLL samples.

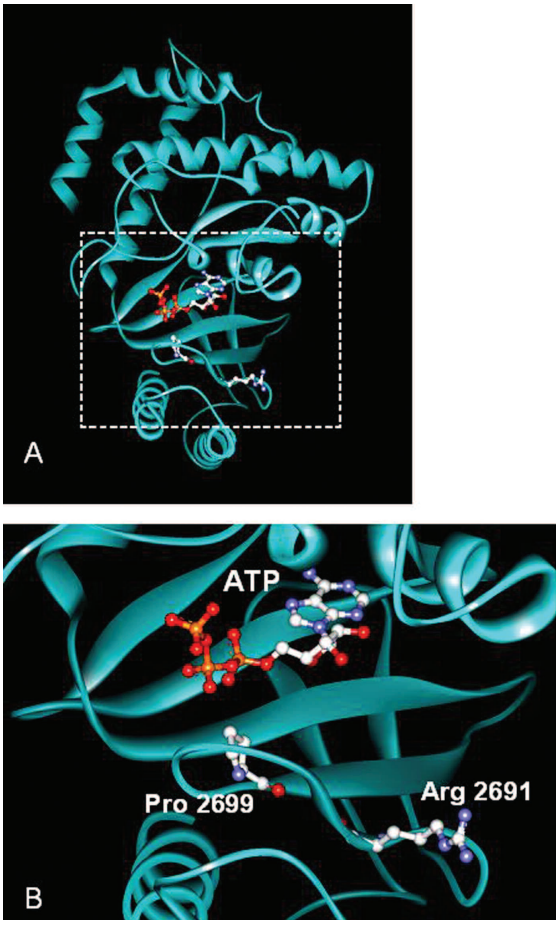

Figure 4. Model of the ATM protein. Ribbon representation of the model of the *ATM* kinase domain (A). The amino acid residues involved by mutations (Arg2691 and Pro2699) and the ATP co-factor are represented by balls and stick. Details of the ATP binding region and the site of mutations are shown in (B).

Furthermore, Weston *et al.*[37] showed that *ATM*-deleted CLL cells exhibit impaired activation of the NFR2-ARE detoxification pathway; consequently, *ATM* mutant cells can be differentially targeted for killing by agents that activate the NFR2-ARE pathway. The targeted approach may provide novel treatment options for otherwise chemoresistant *ATM* mutant tumors and, thereby, additionally reduce morbidity in patients.

When considering *ATM* point mutations, unsupervised analysis revealed that three of five cases with *ATM* aberrations clustered in the same branch, although not tightly: as suggested by Stankovic *et al.*,[38] this might mean that a distinctive signature is not evident prior to DNA damage. At variance, supervised analysis comparing the *ATM*-mutated cases with the remaining samples identified 32 differentially expressed genes. The up-regulated genes included *TGFBR3*, which codifies for a TGFB receptor, *XBP1*, which encodes a transcription factor expressed in almost 80% of estrogen receptor-alpha-positive breast tumors,[39-43] *SRGN*, which encodes for a protein associated with the macromolecular complex of granzymes and perforin, and *EIF4A* and

RBM8A, both involved in regulation of transcription. Among the down-modulated genes, it is worth mentioning *CD180* and *LY86*, which encode two surface molecules associated in a receptor complex (RP105/MD-1) with a role in B-cell recognition and signaling of lipopolysaccharide,[44] *AXIN2*, associated with carcinogenesis in colorectal carcinoma,[45] and *LAMA5* and *SEPT10*, both involved in the pathophysiology of CLL.[46,47]

The peculiar gene expression profile of *ATM*-mutated and deleted patients was confirmed when the analysis was restricted to *IGHV* unmutated cases, suggesting that *ATM* gene alterations alone can induce the gene expression changes.

Our results suggest that both deletions and mutations of the *ATM* gene affect the gene expression profile. However, the genes involved are different in the two groups, with only four genes commonly deregulated in both CLL patients with both mutated and deleted *ATM*, thus indicating that, at the biological level, different mechanisms might be involved in the ATM pathway impairment, but provide a similar adverse clinical effect.

These conclusions are strengthened by the evidence that no specific signatures are associated with *ATM* polymorphisms and are in agreement with the knowledge that *ATM* mutations are pathogenic rather than polymorphic, because *ATM* polymorphisms are not associated with a defect in *ATM*-dependent cellular responses.[18]

The differences observed in gene expression profile among *ATM*-mutated leukemic cells could be the consequence of mutations in different coding regions. In fact, mutations observed in the cases analyzed here occur in different exons, leading to the deregulation of different domains of the ATM protein: given the small number of patients, a comparison of the transcriptional profile of the different mutations was not feasible, although this approach might be useful in order to understand the functional consequence of each mutation.

The ATM protein has a key role in the response to double-stranded DNA breaks, which are potentially harmful to cells; its involvement results in a rapid increase in the kinase activity residing in a protein domain characterized by the PI3K family typical motifs. Bakkenist *et al.* proposed that, in unperturbed cells, ATM proteins associate forming homodimers or higher-order homomultimers devoid of kinase activity.[48] After DNA damage, one ATM molecule phosphorylates serine 1981 on an interacting ATM molecule, enabling dissociation of the latter and phosphorylation of the cellular target.[48]

Two patients carrying the D479T mutation fell in the same cluster of gene expression profiles suggesting that the mutation could play a role in the behavior of the leukemic cells, although no further evidence on the role of this mutation has been reported.

Homology modeling allowed us to locate the sites of R2691C and P2699S mutations in the pocket that binds the ATP co-factor: the amino acid changes associated with both mutations critically impair ATM kinase activity and important biological consequences can be envisioned. Indeed, it has been observed that heterozygous missense mutations dramatically increase the risk of cancer. This phenomenon can be explained by the dominant-negative effect. Specifically, ATM inactive kinase mutants interact with ATM wild-type proteins inhibiting their activation through phosporylation of serine 1981. Hence, these inactive mutants sequester wild-type proteins and inhibit cell responses to the carcinogenic effects of a variety of physical and chemical insults.

Finally, Willmore *et al.* reported that *ATM* mutants display significantly higher activity of DNA-dependent protein kinase (DNA-PK), another pathway involved in the repair of double-stranded DNA breaks, and suggested that DNA-PK inhibition can sensitize *ATM* mutant CLL cells to chemotherapeutics. Their data are consistent with the concept of synthetic lethality, by which tumor cells harboring a DNA repair defect can be killed by targeting the compensatory DNA repair pathway and suggest that a group of patients may benefit from this combination.[49]

In conclusion, this study indicates that *ATM* gene mutations - both point mutations and deletions - occur in a high proportion of cases of newly-diagnosed untreated CLL (24.6%), thus representing the most frequent unfavorable genetic anomaly in CLL. In view of the role played by *ATM* mutations on the behavior of CLL cells and progression of the disease, both deletions and point mutations should be considered in an optimal prognostic stratification of CLL patients and when deciding the management.

Authorship and Disclosures

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