TCL1A and *ATM* are co-expressed in chronic lymphocytic leukemia cells without deletion of 11q

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ABSTRACT

Chronic lymphocytic leukemia is characterized by the accumulation of B cells that are resistant to apoptosis. This resistance is induced by pro-survival stimuli from the microenvironment. *TCL1* and *ATM* are central to the pathogenesis of the disease and associated with more aggressive disease. Their protein products have recently been shown to physically interact in leukemic cells and to impact on NF-κB signaling, which is a key regulator of apoptosis. In the present study we show that *TCL1* and *ATM* are significantly co-expressed and up-regulated in malignant cells compared to non-malignant B cells, and that expression of *TCL1* is partially deregulated by aberrant DNA-methylation. In addition, complex external stimuli induce essentially similar *TCL1* and *ATM* time-course kinetics. In line with a coordinative regulation of NF-κB signaling by *TCL1*, its knockdown induced apoptosis in primary leukemia cells. These findings suggest that both genes functionally cooperate to modulate similar apoptosis-related cellular pathways.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature B lymphocytes in the blood, lymphoid organs and bone marrow and is caused by resistance to apoptosis.[1] This prolonged survival is highly dependent on pro-survival stimuli from the microenvironment including cytokines and stem-cell factor (SCF).[2] The prognosis of CLL is influenced by diverse factors including the mutation status of the immunglobulin heavy chain genes (IGHV) and different cytogenetic aberrations.[1] Loss of genomic material from chromosomal band 11q22-q23 harboring the ataxia telangiectasia-mutated (*ATM*) gene is associated with more rapid disease progression. The serine protein kinase *ATM* is activated by DNA double strand breaks to delay cell cycle and thereby ensure integrity of the genome.[3] *ATM* deficiency is accompanied with genomic instability and a predisposition to lymphoid malignancies.[4] In a number of gene expression profiling analyses, the *ATM* gene showed a small but consistent and significant upregulation in CLL cells (*Online Supplementary Figure S1 and references therein*). Similarly, the strong overexpression of *TCL1* in B cells of mice[5] and men[6] is associated with a more aggressive form of CLL. The *TCL1* oncogene acts as a coactivator of *AKT*,[7] *AP1* and *NF-κB*.[8] It is therefore potentially involved in the resistance to apoptosis that is observed in CLL. Recently, a direct interaction of TCL1 and ATM proteins was reported in CLL in association with activation of the NF-κB pathway.[9] To assess the role of both genes in the resistance to apoptosis, we characterized their expression kinetics in CLL cells stimulated with different microenvironmental support.

Design and Methods

Samples

PBMCs of CLL patients (informed consent; Ethics Committee approval 96/08 Ulm University) and healthy individuals (donors of the German Red Cross, Ulm) were isolated (Ficoll, Biochrom), CD19-selected (MACS Milteny) and controlled for purity by flow cytometry (anti-CD19-FITC, Dako).

Methyl-CpG-Immuno-precipitation (MCIp) - promoter array

Genomic DNA (2 μg) was immunoprecipitated using 30 μg recombinant MBD2–Fc fusion protein coupled to SIMAG protein-A magnetic beads (Chemicell)[10] and hybridized onto custom arrays (eArray, Agilent, G4170-90012 protocol-version.1.0) with promoter tiling from -3.8 to +1.8kbp (60bp oligonucleotides, 30bp non-overlapping spacing, 10bp linker-sequence; GRCh37 hg18).

CLL cell culture

CD19-sorted CLL cells were cultured with bone marrow-derived human ("HS-5") or mouse ("M210B4") stromal cells ($3x10^5$ cells/6-well) or with conditioned medium ("HCM", supernatant of HS-5 cells cultured for 3 days).

Expression analysis

RNA (AllPrep-DNA/RNA-Mini-kit, Qiagen) was first-strand cDNA synthesized (AffinityScript-qPCR-cDNA Synthesis-Kit). QRT-PCR

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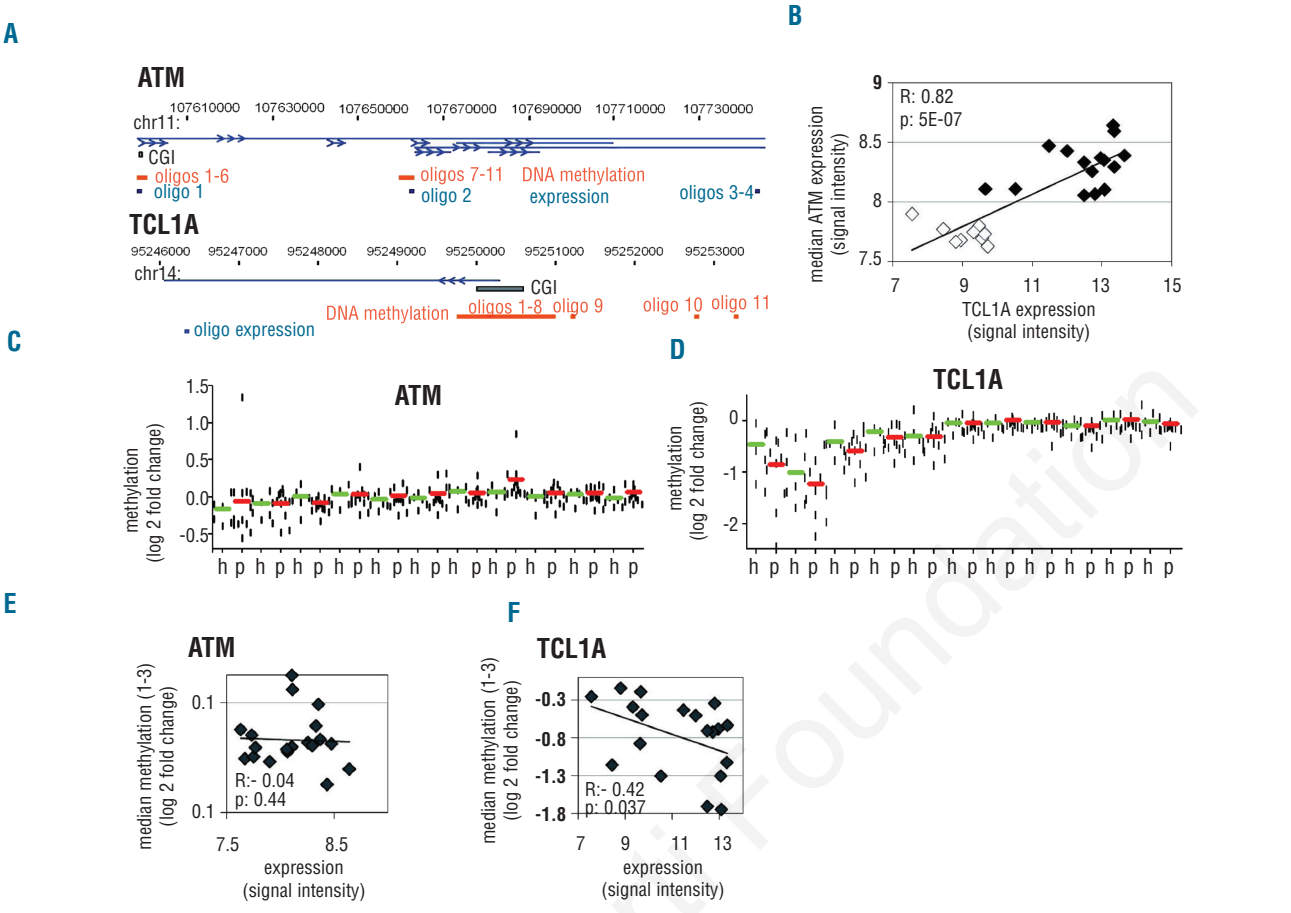

Figure 1. ***TCL1A*** **expression strongly correlates with expression of** ***ATM*** **in malignant and non-malignant B cells and might partially be deregulated by CGI DNA-hypomethylation. (A) Schematic representation of the genomic organization of** ***ATM*** **and** ***TCL1A*** **with the corresponding CpG Islands (CGI, gray boxes). The chromosomal location according to genome build hg18 is given above the transcripts depicted as blue horizontal lines, direction of transcription indicated by arrowheads. Orange/blue boxes represent the location of oligonucleotides on the promoter arrays used for DNA-methylation analysis (*DNA-methylation*) and the expression arrays (*expression*) and are numbered according to their genomic localization and order in panels C and D. (B)** ***TCL1A*** **expression significantly correlates with expression of** ***ATM*** **(median of four oligonucleotides, *see panel A*) in CLL cells (black diamonds) and non-malignant B cells (white diamonds). Pearson's correlation coefficient is denoted by R and significance was tested using R/sqrt[(1-R²)/(N-2)]. (C-D) Median DNA-methylation levels in non-malignant B cells (*h*, green line represents median levels) and CLL cells (*p*, red line represents median levels) in the promoter of** ***TCL1A*** **(D) and** ***ATM*** **(C). (E) Median DNA-methylation levels at the** ***ATM*** **promoter do not correlate with median expression levels of** ***ATM*****. (F) DNA hypomethylation at the 5´end of the** ***TCL1A*** **promoter represented by oligos 1-3 shows a significant inverse correlation with** ***TCL1A*** **expression in CLL cells, suggesting a functional connection.**

was performed with Absolute-QPCR-SYBR-Green-ROX-Mix (Thermo-Scientific) containing 70nM primers (*Online Supplementary Table S1*) with 7300-Real-time-PCR-System (Applied Biosystems) at 15 minutes 95°C, 40 cycles: 15s 95°C; 30s 60°C. Expression arrays: Illumina-Human-Sentrix-12-BeadChip.

Modeling and statistics

For analysis and visualization of previously published gene expression data, Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used. For details of network modeling please *see Online Supplementary Design and Methods*. In brief, genes were grouped into clusters according to their expression kinetics using Partitioning Around Medoids (PAM) clustering method to yield an appropriate number of genes. The resulting medoids represent genes corresponding to network nodes. Networks were estimated using a dynamic Bayesian network approach. The analysis employs a Markov Chain Monte Carlo algorithm for obtaining posterior edge probabilities of the network.

SiRNA transfection

We transfected 5x10⁶ CLL-PBMCs using 1 µg siRNA targeting *TCL1A* (Silencer-Select-Pre-designed-and-Validated siRNA, Ambion) and 100µl B-cell solution-B using program U-015 (Amaxa NucleofectorII). ON-TARGETplus SMARTpool siRNA, MCL-1 (Dharmacon) and Silencer-Negative-Control #1 (Ambion) were used as controls. After transfection, cells were cocultured with HS-5 cells (2.6x10⁴ cells/ 24-well). CLL cell survival was analyzed by staining 7-AAD, Annexin V and propidium iodide (PI) and CD19-APC using FACSCalibur flow cytometer (BD Biosciences).

Results and Discussion

TCL1A and *ATM* (Figure 1A) are central players in the pathogenesis of CLL. We therefore studied transcription of TCL1A and ATM and discovered a strikingly strong correlation of their expression levels in primary cells (Figure

1B). In order to rule out confounding effects caused by deletion of the critical region in 11q22-q23 harboring the ATM and the miR34b-5p genes that target TCL1A,[11,12] only CLL cells without cytogenetic aberrations or only harboring a single deletion of 13q14.3 were analyzed (Table 1). To ask whether both genes are synchronously deregulated via epigenetic aberrations we determined DNA-methylation levels at *ATM* and *TCL1A* promoters using Methyl-CpG Immonuprecipitation (MCIp) followed by hybridization onto custom promoter arrays (position of oligonucleotides: Figure 1A). Within the *ATM* promoter no aberrant DNA-methylation was detected in CLL samples (n=13) compared to non-malignant B cells from age-matched healthy controls (n=6; Figure 1C). However, the *TCL1A* promoter displayed hypomethylation in CLL cells (Figure 1D) significantly correlating with *TCL1A* transcriptional upregulation (Figure 1F). These results are in agreement with previous reports[13,14] and suggest that the small but significant *ATM* overexpression in CLL (*Online Supplementary Figure S1 and references therein*) is independent of promoter DNA-methylation in CLL cells that do not carry deletion of 11q22-q23 (Figure 1E). In contrast, *TCL1A* might partially be up-regulated in CLL cells by DNA hypomethylation (Figure 1F).

Given the significant coexpression of *TCL1A* and *ATM* we investigated whether both genes display similar expression kinetics when CLL cells are exposed to different stimuli *in vitro*, both by murine and human cells.[15] Therefore we subjected primary CLL cells to different culturing conditions, and both *TCL1A* and *ATM* were synchronously downregulated in CLL cells upon culture (Figure 2 A and B). This is unlikely to be caused by miR34a/b localized in 11q and targeting *TCL1A*,[12] as these miRs are not dysregulated upon coculture.[17] While stimulation with nurse-like cells has been shown not to impact on expression of TCL1A,[18] here expression levels of *ATM* and *TCL1A* consistently increased after 8 h of culture independent of the stimulus applied. Coexpression dynamics of both genes were validated in 6 CLL patient samples exposed to the different culture conditions at serial timepoints using qRT-PCR (Figure 2C-F). Strikingly, we observed a highly similar expression pattern for *ATM* and *TCL1A* in all patient samples with exception of CLL#5 in HS5 coculture (Figure 2F). CLL#5 had no different clinical history or cytogenetic aberrations compared to the remaining patient cohort (Table 1).

TCL1A and *ATM* were among the 8 most significantly deregulated genes between stimulated and non-stimulated conditions (*Idler, Bhattacharya et al., manuscript in preparation*). This allowed us to compare their coregulation to the 6 remaining candidate genes using dynamic Bayesian networks, ranking all expression profiled genes according to i) deregulation in the stimulated *versus* non-stimulated culture conditions and ii) coregulation upon different culture conditions (see supplementary materials and methods for detailed description of analysis). From the top 0.5% ranked candidates (n=243) all genes functionally associated with GO-terms "apoptosis", "cell death" and "survival" in IPA (Ingenuity Systems, www.ingenuity.com; n=66 genes) and DAVID[19] (n=53 genes) were selected. Thirty-five genes present in both databases were grouped via gene expression kinetics into 8 clusters using Partitioning Around Medoids (PAM) clustering to yield an appropriate number of genes for network modeling. Medoids that best represent kinetics of these clusters (*HSPA1B, ZFP36, VHL, ATM, NFKB1, INPP5D, FOS, ATM* and *TCL1A*) were employed as nodes in a dynamic Bayesian network analysis (Figure 2G). Intriguingly, of all gene-interrelations deducted from expression kinetics, the connection of *ATM* and *TCL1A* was the strongest (Figure 2G, H). This suggested a coregulation of both genes in primary CLL cells when applying different stimuli. The Markov Chain Monte Carlo algorithms underlying the network estimation were initiated with either a random or an empty network, which yielded similar results (Figure 2H) and showed that the estimated networks are stable.

Towards understanding the coregulation of *TCL1A* and *ATM* in CLL, sets of transcription factors (TFs) bound at the gene bodies of the most deregulated genes *HSAP1B, ZFP36, NFKB1, VHL, INPP5D, FOS, TCL1A* and *ATM* were identified in publicly available data of chromatin immunoprecipitations from 95 different cell lines and culture conditions[20] (visualization and analysis via the University of California Santa Cruz (UCSC) genome browser: *genome.ucsc.edu*). Most transcription factors were bound at the *ATM* gene (75 TFs), which was therefore used as reference. All genes showed a significant overlap in the bound TFs compared to *ATM* (on average 70%) but also carried specific TFs that were not detectable at the *ATM* gene (Figure 2 I, red portion). However, all of the TFs binding to TCL1A were also detectable at the *ATM* gene (Figure 2 I, right two columns). While this full overlap of the set of transcription factors is suggestive that *TCL1A* is

Table 1. Characteristics of samples from untreated CLL patients and healthy probands that were used for the indicated analyses.

MCIp-promoter array/expression array		#
Karyotype	no cytogenetic aberrations	6
	del 13q14	7
IGHV	mutated	9
	unmutated	4
Gender	male	9
	female	4
Median age (range)	55 (40-73)	
expression qRT-PCR (years)		#
Karyotype	no cytogenetic aberrations	2
	del 13q14	5
IGHV	mutated	5
	unmutated	2
Gender	male	4
	female	3
Median age (range)	53 (46-67)	
siRNA knockdown (years)		#
Karyotype	no cytogenetic aberrations	2
	del 13q14	3
IGHV	mutated	4
	unmutated	1
Gender	male	4
	female	1
Median age (range) (years)	71 (55-82)	
Healthy individuals		
MCIp-promoter array/expression array		#
Gender	male	9
	female	4
Median age	56 (30-59)	

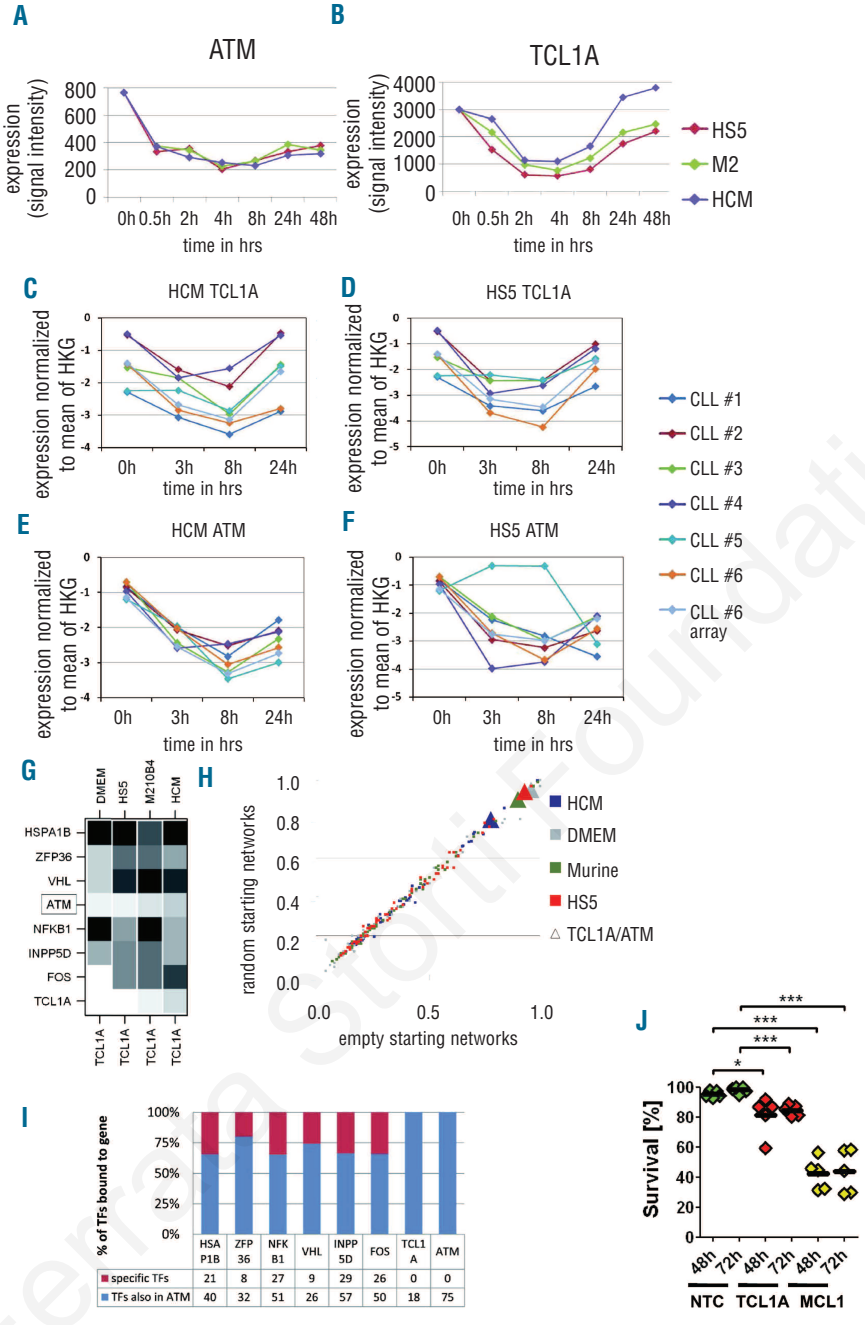

Figure 2. *TCL1A* and *ATM* are co-expressed in CLL cells upon *in vitro* culture with different stimuli. *ATM* (A) and *TCL1A* (B) expression was detected by Illumina Human Sentrix-12 BeadChip expression arrays at different time points (0h, 0.5h, 2h, 4h, 8h, 24h and 48h) in a single patient cultured with HS5 conditioned medium (*HCM*), with human stromal cells (*HS5*) or murine M210B4 stromal cells (*M2*). (C-D) Expression of *TCL1A* was quantified with qRT-PCR in CLL cells from 6 CLL patients (CLL#1-6, with CLL#6 analyzed also by microarray) cultured with conditioned medium (*HCM*, C) or co-cultured with human stromal cells (HS5, D). (E-F) Expression of *ATM* as described in (C-D). (G) Posterior edge probabilities of dynamic Bayesian network models indicated by gray-shading (black and white corresponding to probability 0 and 1, respectively) between network node *TCL1A* and the most deregulated genes. *ATM* (marked by gray box) displays the highest edge probability (strongest influence of *TCL1A*). (H) Scatterplot of posterior edge probabilities for dynamic Bayesian networks of the 8 genes most deregulated upon *in vitro* culture. Using a random starting network as initial situation (y-axis) or an empty starting network (x-axis) resulted in similar probabilities, showing that the derived Bayesian networks are stable. Interrelations of *TCL1A* and *ATM* displayed the highest probabilities (triangles, top right corner). (I) Analysis of transcription factor binding using the publicly available ENCODE immunoprecipitation data of 95 cell lines/conditions showed that all genes (x-axis) are bound by specific transcription factors (*TFs*, % on y-axis, absolute numbers are given below the graph) that do not bind to *ATM* (red portion) with the noticeable exception of *TCL1A*. *TCL1A* is only bound by TFs that can also be detected at the *ATM* gene. This suggests that both *TCL1A* and *ATM* might be regulated by similar pathways leading to coregulation of these genes in CLL cells. We analyzed the complete gene body including 200 bp of upstream and downstream sequence. (J) *TCL1A* impacts on the resistance to apoptosis in CLL cells. Five freshly isolated primary CLL patient samples were transfected with random siRNA (*NTC*: no target control) or siRNAs targeting *TCL1A* or MCL1 (used as positive control)[16] and co-cultured with HS5 cells seeded two days before. After 48h and 72h, the percentage of surviving CLL cells was measured by flow cytometry after staining with 7AAD, Annexin V/PI and CD19 APC and normalized to mock-transfected cells. Statistics were performed using unpaired t-test (***P<0.001; **P=0.001-0.01; *P=0.01-0.05).

the target of similar signaling pathways as *ATM*, the analyzed data are not specific to B or CLL cells and should be viewed with caution.

In line with a functional connection of *TCL1A* and *ATM*, a direct interaction of their gene products has recently been shown in CLL and linked to activated NF-κB signaling,[9] a pathway centrally involved in CLL pathogenesis[21] and apoptosis.[22] We therefore tested whether knockdown of *TCL1A* causes apoptosis of primary CLL cells (n=5). In comparison to a non-target control siRNA and a siRNA targeting *MCL1*[23] used as positive control, knockdown of oncogenic *TCL1A* caused a significant reduction in CLL cell survival. This suggests a role of *TCL1A* in the resistance to apoptosis described for CLL cells[23] (Figure 2J; for validation see *Online Supplementary Figure S2*).

In summary, *ATM* and *TCL1A* are significantly coexpressed and display similar kinetics when exposed to different external stimuli. This suggests that both genes are involved in similar cellular pathways. In line with previous reports that *TCL1A* knockdown reduces NF-κB activation,[9] knockdown of *TCL1A* in primary CLL cells induced apoptosis. The joint deregulation of *TCL1A* and *ATM* might therefore contribute to the massive accumulation of malignant cells in CLL patients.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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