Methods (online version)

Chronic lymphocytic leukemia patients and sample processing

The study was performed on peripheral blood mononuclear cells (PBMNC) of 140 CLL patients followed at the Department of Internal Medicine – Hematology and Oncology, University Hospital Brno. CLL diagnosis was based on standard immunophenotypic criteria.[1] All samples were processed with written informed consent in accordance with the Declaration of Helsinki under protocols of the University Hospital Brno (Ethics Committee approval NS10439-3).

PBMNC were isolated using Histopaque-1077 (Sigma). In samples with lower proportion of CLL cells, the enrichment for B-lymphocytes was performed by RosetteSep (Stem Cell Technologies). The leukemic cells proportion (CD5+/CD19+) was determined by flow cytometry and exceeded 80% in all samples. Basic characteristic of the cohort is summarized in Table 1 and therapy overview is stated in *Online Supplementary Table S1*). The selection of samples was intentionally biased towards high-risk CLL patients harboring *TP53* defect and/or 11q- and/or unmutated *IGHV* locus in order to assess functional impact of *ATM* mutations in such adverse cohort.

Analysis of standard prognostic factors and TP53 mutations

Deletions at the 11q22-23 (*ATM*) and 17p13 (*TP53*) loci were detected by I-FISH using locus-specific probes (Abbott Vysis), according to manufacturer instructions. At least 200 interphase nuclei per slide were evaluated using LUCIA KARYO/FISH/CGH imaging system (Laboratory Imaging). The 5% cut-off level for the *TP53* and *ATM* deletions detection was determined by analysis of 15 samples obtained from healthy bone-marrow donors. PCR and direct sequencing were used to analyze the *IGHV* rearrangements and mutation status. *TP53* mutations were identified by the yeast functional analysis (FASAY)[2] followed by

mutated templates sequencing or using a direct sequencing of genomic DNA (complete coding region, exons 2-11) according to principles and procedures we previously published.[3]

Identification of ATM mutations by resequencing microarray

Custom resequencing microarray (Affymetrix GenChip CustomSeq Resequencing Array platform, Affymetrix) was used to detect *ATM* mutations. The array was designed to capture 1-nt substitutions in all 62 coding exons and adjacent splicing sites. Probes for small nucleotide insertions and deletions could not be included on chip due to their unpredictable positions and range. The resequencing principle is based on allele-specific hybridization. Each examined base was represented by eight probes (25 bases long) to cover alterations in both strands. The hybridization of fluorescently labeled DNA fragments to particular positions determined the nucleotides in sequence by emitting a signal with ability to distinguish between the homozygous and heterozygous state.

Genomic DNA was isolated from CLL cells using DNeasy Blood&Tissue Kit (Qiagen). The resequencing procedure was carried out according to manufacturer protocol (Affymetrix GeneChip Custom Resequencing Array Protocol). Briefly, the long-range PCR (using LA TaKaRa Polymerase, Takara) was done in 19 reactions covering the whole *ATM* gene (primer sequences are available on request). All PCR amplicons were quantified using PicoGreen Assay (Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen), pooled in equimolar amounts, and the pool of PCR products was purified (QIAquick PCR Purification Kit, Qiagen). The fragmentation (modification contrary to protocol: 0.05U of fragmentation enzyme, 10 min reaction), labeling with biotin and hybridization to the array, were performed using Affymetrix GeneChip Resequencing Reagent Kit. Finally, arrays were washed and stained using a FS 450 fluidic station and scanned with scanner GCS3000 7G (Affymetrix).

Final sequence data was acquired using The GeneChip Sequence Analysis Software (GSEQ) processing fluorescence intensity files. GSEQ automates the base calling process using an algorithm with adjustable settings. To obtain the highest call rate without increasing the false negative rate, we were evaluating Quality Score Threshold (QST) and Base Reliability Threshold (BRT) and finally determined QST = 3 and BRT = 0.

Searching for ATM mutations by direct Sanger sequencing

Sanger sequencing was used to: (i) confirm *ATM* alterations detected by array; (ii) identify other mutations indicated by functional test and/or western blot (WB) by screening of all 62 coding exons and adjacent splicing sites. Concerning confirmation of variants detected by array, we observed that variant calls with more than 1 "no call" (N) at a particular position among other samples were always false positive. Thus, all alterations further confirmed by Sanger sequencing exhibited only 0-1 of N at the position. PCR products (primer sequences available on request) were sequenced using 3130xl Genetic Analyzer (Applied Biosystems). Final sequences were analyzed by software Geneious 4.8.2 (Biomatters).

All confirmed sequence variations underwent searching for in appropriate databases of single nucleotide polymorphisms (SNPs) (National Institute of Health database of SNPs) and mutations (Sanger Institute database; HGMD database).

Analysis of ATM mutations by deep sequencing

The pyrosequencing on the GS Junior 454 platform (Roche) was performed according to the manufacturer protocol. Shortly, genomic DNA was extracted using DNeasy Blood&Tissue Kit (Qiagen). PCR was conducted with proof-reading Q5 Polymerase (New England BioLabs) using primers composed of the following parts from the 5´-end: (i) 25-mer corresponding to the adaptors A and B required by the 454 sequencing system; (ii) multiplex

identifiers of 10 bp used to barcode the different samples; (iii) *ATM* specific sequence. PCR products were analyzed by Agilent 1000 DNA Kit on Bioanalyzer 2100 (Agilent). Each amplicon was individually purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using the Qubit dsDNA HS assay (Life Technologies). Amplicon pool was generated by combining 1 x 10^9 molecules of each PCR product in equimolar ratio. Emulsion PCR of the combined library was carried out using the GS Junior Titanium emPCR Kit (Lib-A). The copy per bead ratio used was 1:2. The library was sequenced on Roche GS Junior with Titanium reagents (Roche). One positive and at least one negative control for each variant (represented by corresponding exons with known *ATM* status from resequencing analysis) were included in the experiment.

Sequence alignment and variant detection was performed using the GS Amplicon Variant Analyzer software version 2.5 (Roche). For the analysis of searched variants bidirectional reads and forward/reverse ratio (number of forward/reverse reads with change) were evaluated. Only the variants having this ratio between 0.5-2 were considered as real substitutions. All controls showed the expected output and the forward/reverse ratio in two confirmed alterations was 1.4.

Western blotting

Antibodies anti-ATM (rabbit mAb D2E2; dilution 1:1000), anti-ATM-Ser1981 (rabbit mAb D6H9; 1:2000), anti-p53-Ser15 (mouse mAb 16G8; 1:1000), and anti-β-actin (rabbit mAb 13E5; 1:1000) (all Cell Signaling Technology) were used for corresponding protein level detection. Total p53 level was detected by DO-1 mouse mAb (gift from Dr. Vojtesek, MMCI, Brno; supernatant 1:40). PBMNC were lysed in RIPA buffer at 4 ºC for 30 min and used amount of protein lysates was following: 30 µg (β-actin), 50 µg (ATM-Ser1981), 75 µg (total p53), and 100 µg (total ATM and p53-Ser15). The proteins were run on NuPAGE

Novex 3-8% Tris-Acetate Gel (Invitrogen) or 10% TBE-PAGE gel and subsequently transferred to nitrocellulose membrane (0.2 μm pores, Bio-Rad). Secondary antibodies were HRP-conjugated (DakoCytomation). Protein level detection was done using Lumi-light Western Blotting Substrate (Roche).

Induction of p53-downstream target gene expression after fludarabine and doxorubicin (functional test) or after IR exposure

Vitally frozen cells were thawed and subjected to ionizing radiation (IR) (5 Gy in total, 0.3 Gy/min) or treated with fludarabine (Bayer-Schering, 3.6 μg/ml) or doxorubicin (Teva, 0.25 μg/ml). Cells were seeded in 6-well plates (2.5×10^7 cells per well, volume 5 ml) and harvested after 2, 10 and 24 h in case of IR or after 24 h drug exposure. Lysates for total RNA isolation were prepared using TriReagent (Molecular Research Center, Inc.).

Total RNA was isolated by the RNeasy Mini Kit with the DNase I digestion (Qiagen). RNA (500 ng) was reverse transcribed using Superscript II RT (Invitrogen). Real-time PCR assay was performed using TaqMan technology and 7300 Real-Time PCR System (Applied Biosystems). Primer and probe set was specific for the *CDKN1A* (*p21)* in case of functional test, and also for *BBC3* (*PUMA), BAX*, and *GADD45* genes in the subsequent p53 pathway analysis (TaqMan Gene Expression Assays, Applied Biosystems). Geometric mean of *TBP* and *HPRT1* cycle threshold (C_t) values served as an internal standard. Sequence Detection Software (version 1.3.1; Applied Biosystems) was used to analyze the fluorescence emission data after real-time PCR. Sample's C_t values were subjected to $2^{-\Delta\Delta Ct}$ analysis.

Viability testing after doxorubicin and fludarabine administration

Vitally frozen primary CLL cells were thawed, seeded in 96-well plates in quadruplicates (5×10^5 cells per well, volume 200 μl) and subjected to 48 h doxorubicin and

fludarabine exposure at four different concentrations (Figure 3A-B). The cell viability was assessed by the metabolic WST-1 assay (Roche) using spectrophotometer 1420 Multilabel Counter Victor (PerkinElmer).

Statistical evaluation

The comparison of p53-downstream genes induction between individual genetic groups was assessed by Kruskal-Wallis ANOVA test (after drug administration) or Mann-Whitney U test (after IR exposure). Cell viability testing was done using two-factorial ANOVA with subsequent Dunnett post-hoc test. The time to first treatment (TTFT) and progression-free survival (PFS) analyses were performed by creating and statistically evaluating Kaplan-Meier curves using the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

References:

1. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 2008;111:5446–56.

2. Flaman JM, Frebourg T, Moreau V, Charbonnier F, Martin C, Chappuis P, et al. A simple p53 functional assay for screening cell lines, blood, and tumors. Proc. Natl. Acad. Sci. U.S.A. 1995;92:3963–7.

3. Trbusek M, Smardova J, Malcikova J, Sebejova L, Dobes P, Svitakova M, et al. Missense mutations located in structural p53 DNA-binding motifs are associated with extremely poor survival in chronic lymphocytic leukemia. J. Clin. Oncol. 2011;29:2703–8.

Supplementary data (Tables and Figures)

Table S1. Overview of therapy status at the time of *ATM* mutation investigation in the studied CLL patients.

	Number
Patients	140
Untreated	77 (55%)
Treated	63 (45%)
Median of therapies	2 (range 1-5)
Pure chemotherapy	25
Immunotherapy*	33
Radiotherapy	1
Data unknown	4

* Therapy involving monoclonal antibodies (rituximab, alemtuzumab, ofatumumab) either on their own or in combination with chemotherapy or glucocorticoids.

Table S2. Evaluation of *ATM* alterations detected by resequencing array using deep sequencing (GS Roche Junior). Seventeen substitutions identified by resequencing microarray but unconfirmed by direct Sanger sequencing were analyzed, and only two of them were found as real substitutions present in a small number of reads (samples 14 and 15). Two alterations could not be evaluated due to insufficient coverage. In total, resequencing array showed 30% (13/44) of false positive calls.

Sample	**Nucleotide change**	**Amino acid change**	**Exon**	**Original nucleotide (%)**	**Variant nucleotide (%)**	**Number of reads**	**Variant on resequencing array**
1	A2107G	703	15	100	0	3853	FP
2	A2107G	703	15	100	0	4272	FP
3	T3081G	1027	23	100	0	4374	FP
4	T3081G	1027	23	100	0	2274	FP
5	T3081G	1027	23	100	0	2230	FP
6	T3081G	1027	23	100	0	3258	FP
7	T3081G	1027	23	100	0	4193	FP
8	T3128G	1043	23	100	0	3258	FP
9	T3162G	1054	24	100	0	1990	FP
10	A4426C	1476	31	100	0	7001	FP
11	T4630G	1544	33	100	0	5286	FP
12	A5560C	1854	39	100	0	5060	FP
13	A6102C	2034	44	100	0	5352	FP
14	A7310T	2437	52	87,07	9,89*	5745	real
15	A7318G	2440	52	84,31	15,69	5881	real

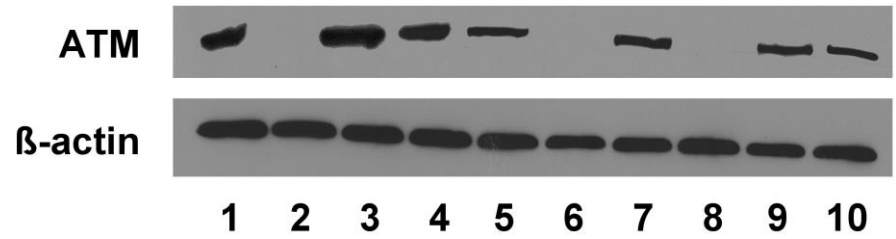
FP – false positive; * no nucleotide detected in 3.05% of reads

Table S3. Polymorphisms detected by resequencing array in CLL patients.

Order	Nucleotide change	Amino acid change	Exon	dbSNP (rs ID)	Number of patients	Frequency (%)	Functional status
1	C146G	Ser49Cys	5	rs1800054	4	4	functional
2	A670G	Lys224Glu	9	rs145053092	1	1	functional
3	C735T[1]	Val245Val	9	rs3218674	3	3	functional
4	T2572C	Phe858Leu	19	rs1800056	1	1	functional
5	C3161G	Pro1054Arg	24	rs1800057	3	3	functional
6	C4258T	Leu1420Phe	31	rs1800058	4	4	functional
7	C4578T	Pro1526Pro	32	rs1800889	7	7	functional
8	G5557A	Asp1853Asn	39	rs1801516	24	22	functional
9	A5558T	Asp1853Val	39	rs1801673	3	3	functional
10	T5793C	Ala1931Ala	41	rs3092910	1	1	functional
11	A5975C	Lys1992Thr	42	rs150757822	1	1	functional
12	G6067A	Gly2023Arg	43	rs11212587	2	2	NA
13	G6860C	Gly2287Ala	49	rs1800061	1	1	NA

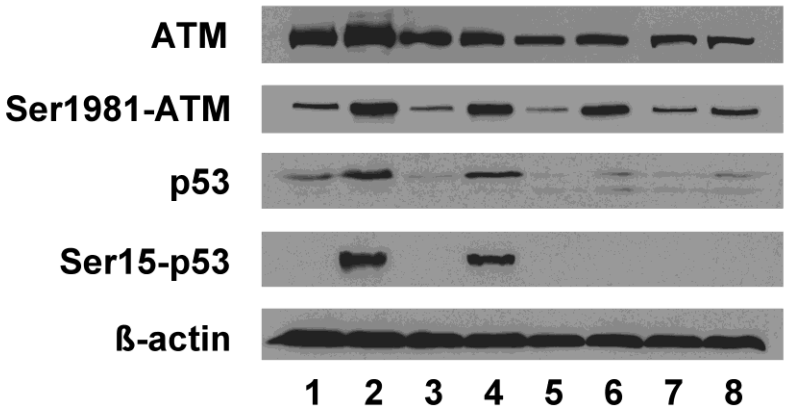
[1] previously reported (Grønbaek et al., Blood 2002,100:1430-7; Laake at al., Hum Mutat 2000,16:232-46) alternative splicing of exon 9 detected in small proportion of molecules, which was also confirmed in our study (data not shown)

Figure S1. Total ATM protein level of analyzed patients (illustrative western blot).



Legend: 1 – positive control (SUDHL-4 cell line), 2 – negative control (lymphoblastoid cell line from patient with AT), 3-10 – CLL patient samples; samples in lines 6 and 8 harbor ATM dysfunction (patients P3 and P8 in Table 2, respectively).

Figure S2. *ATM* mutations lead to abrogation of ATM function towards p53 activation. CLL cells from four patients were subjected to IR (5 Gy in total, 0.3 Gy/min) and analyzed by WB after 1 h incubation. The wt sample and the sample with a sole 11q- exhibited the expected ATM autophosphorylation on Ser1981 and p53 phosphorylation on Ser15 accompanied by the p53 protein accumulation. By contrast, one of two *ATM*-mutated samples (ATM-mut-1) showed obviously diminished autophosphorylation and both *ATM*-mutated samples lost all activity towards p53 activation.



Legend: 1, 2 – wt sample without and after IR, respectively; 3, 4 – sample with sole 11q- without and after IR, respectively; 5, 6 - ATM-mut-1 (11q-/missense mutation Leu2427Pro; P9 in Table 2) sample without and after IR, respectively; 7, 8 - ATM-mut-2 (wt/missense mutation Arg3008Cys with predicted DNE; P13 in Table 2) sample without and after IR, respectively.

Figure S3. Receiver operation characteristic (ROC) analysis for the proposed ATM functional test (A), and distribution of *p21* induced expression after doxorubicin treatment in the tested cohort (B).

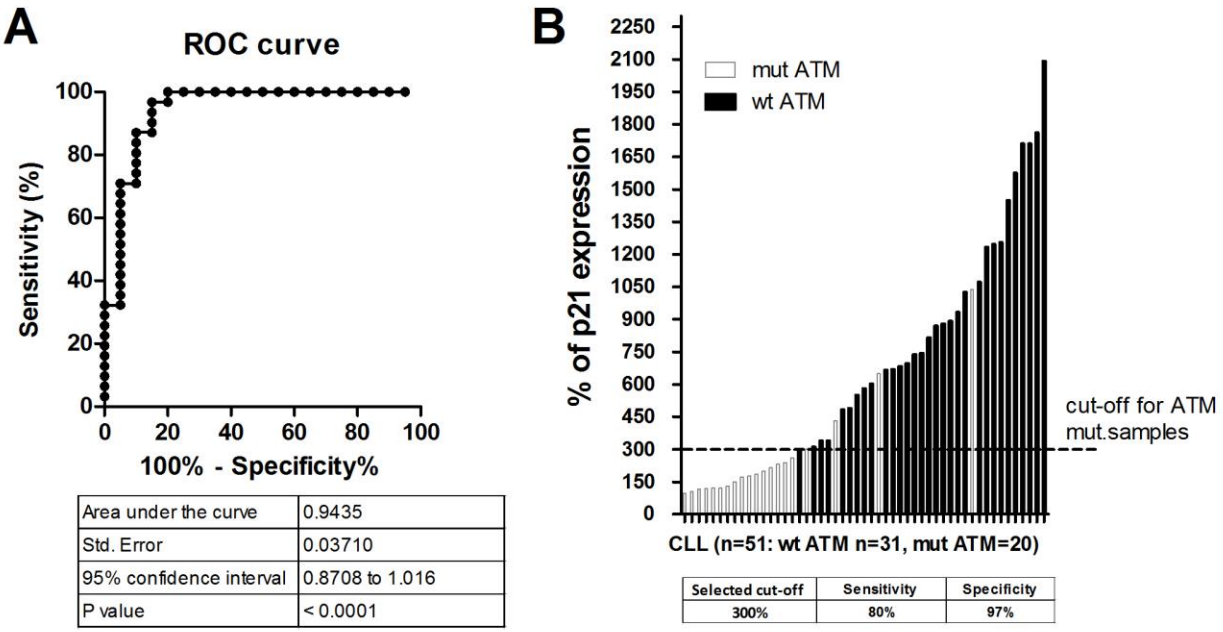


Figure S4. The functional test sensitivity according to the proportion of mutated cells. *ATM*-mutated sample (P13 in Table 2) had been diluted with *ATM*-wt cells, the mixture was treated with doxorubicin for 24 h, and the *p21* expression assessed by real time PCR. The dilution containing 80% of mutated cells indicated mutation presence in the settings of 300% threshold.

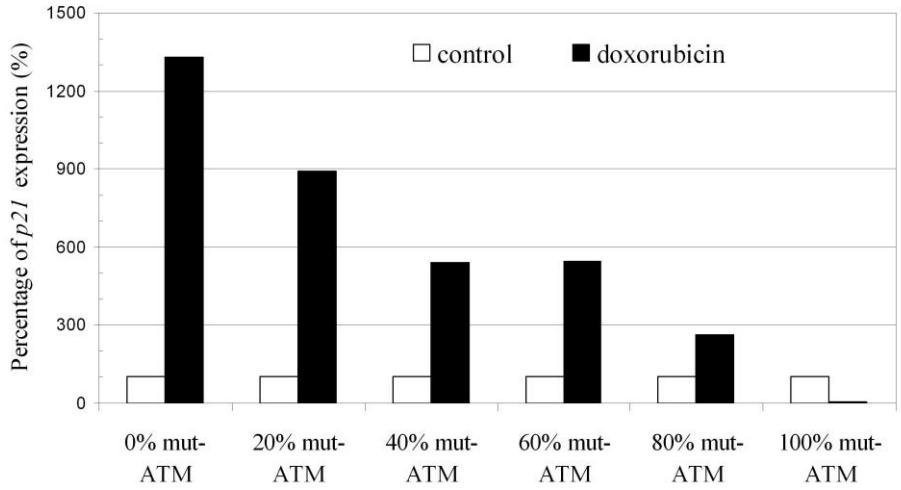
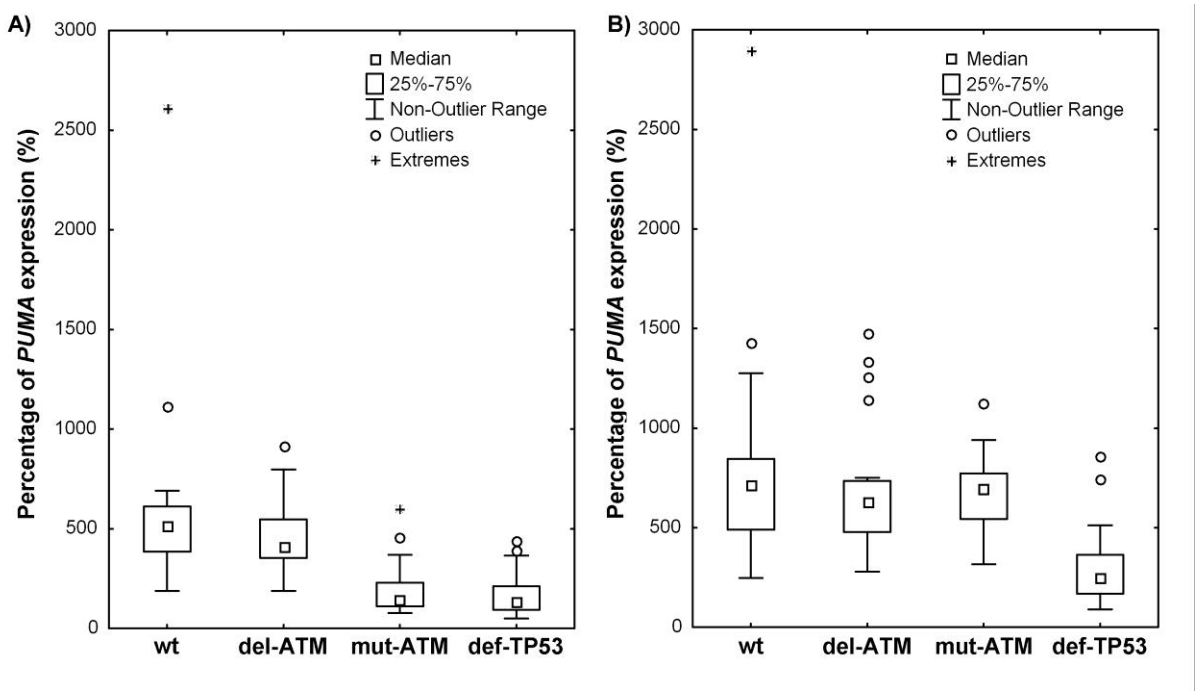
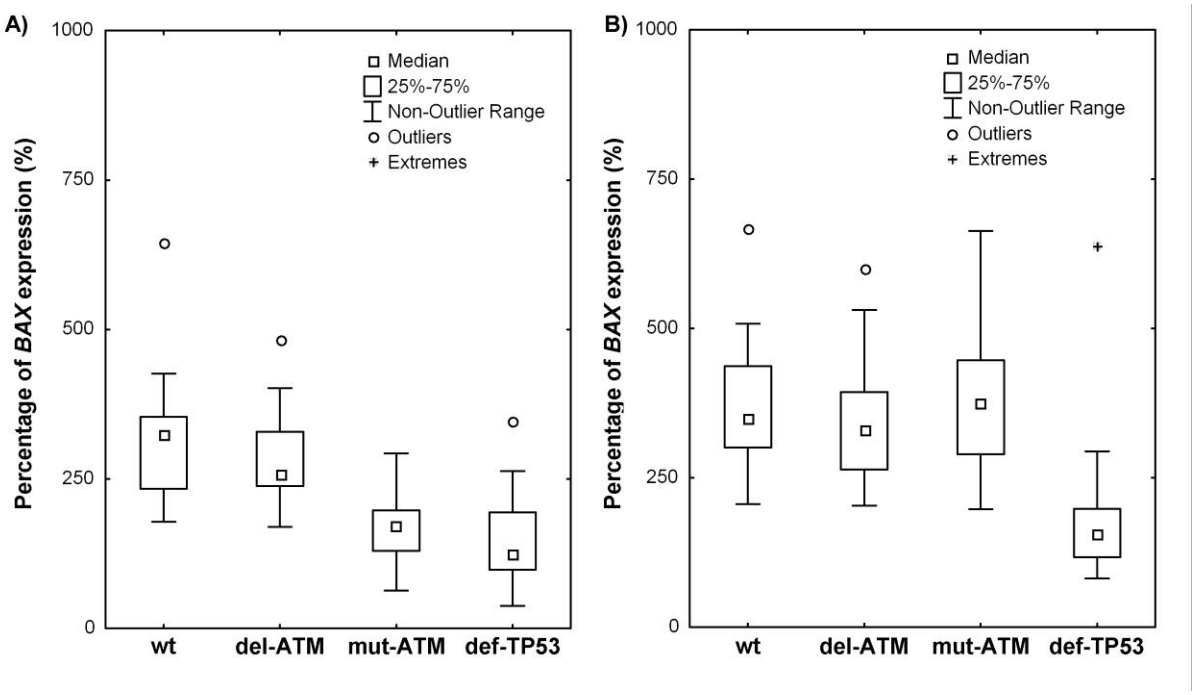


Figure S5. The p53-downstream gene (*PUMA*, *BAX*, *GADD45*) expression induction in individual genetic groups after doxorubicin and fludarabine exposure.

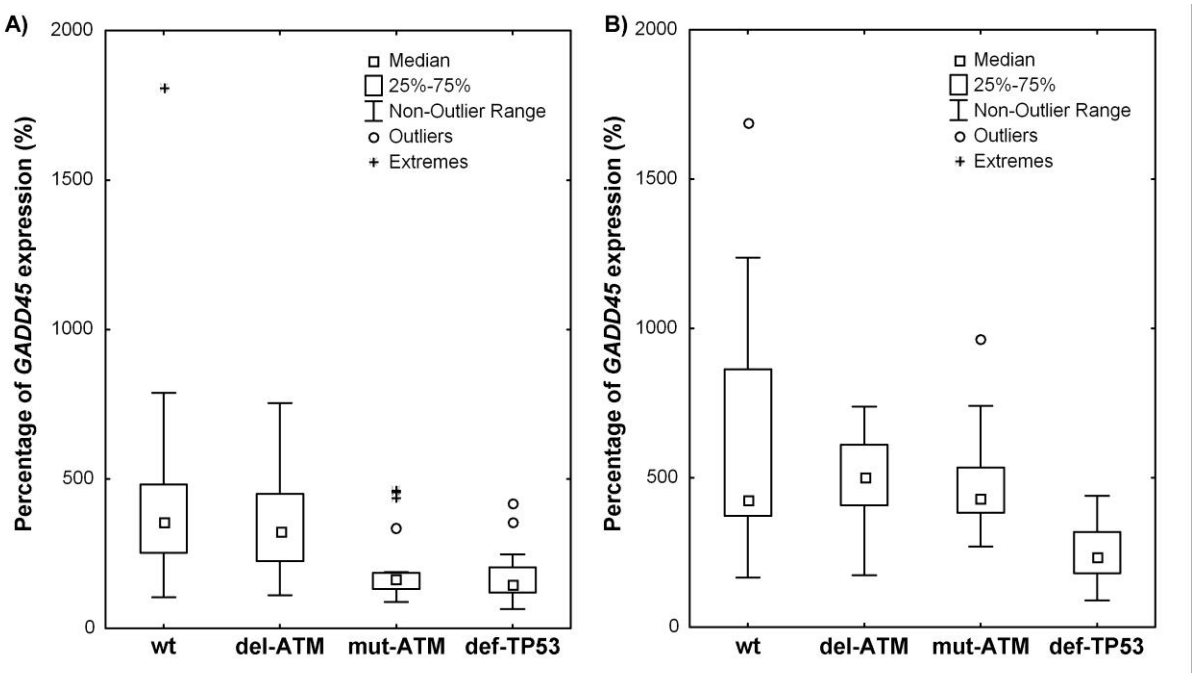
Medians of individual gene expression and the significance related to wt group are listed below the figures.



(A) after doxorubicin treatment: wt: 516%; del-ATM: 415%, non-significant (NS); mut-ATM: 147%, P<0.001; def-TP53: 137%, P<0.001.
(B) after fludarabine treatment: wt: 712%; del-ATM: 628%, NS; mut-ATM: 696%, NS; def-TP53: 245%, P<0.001.



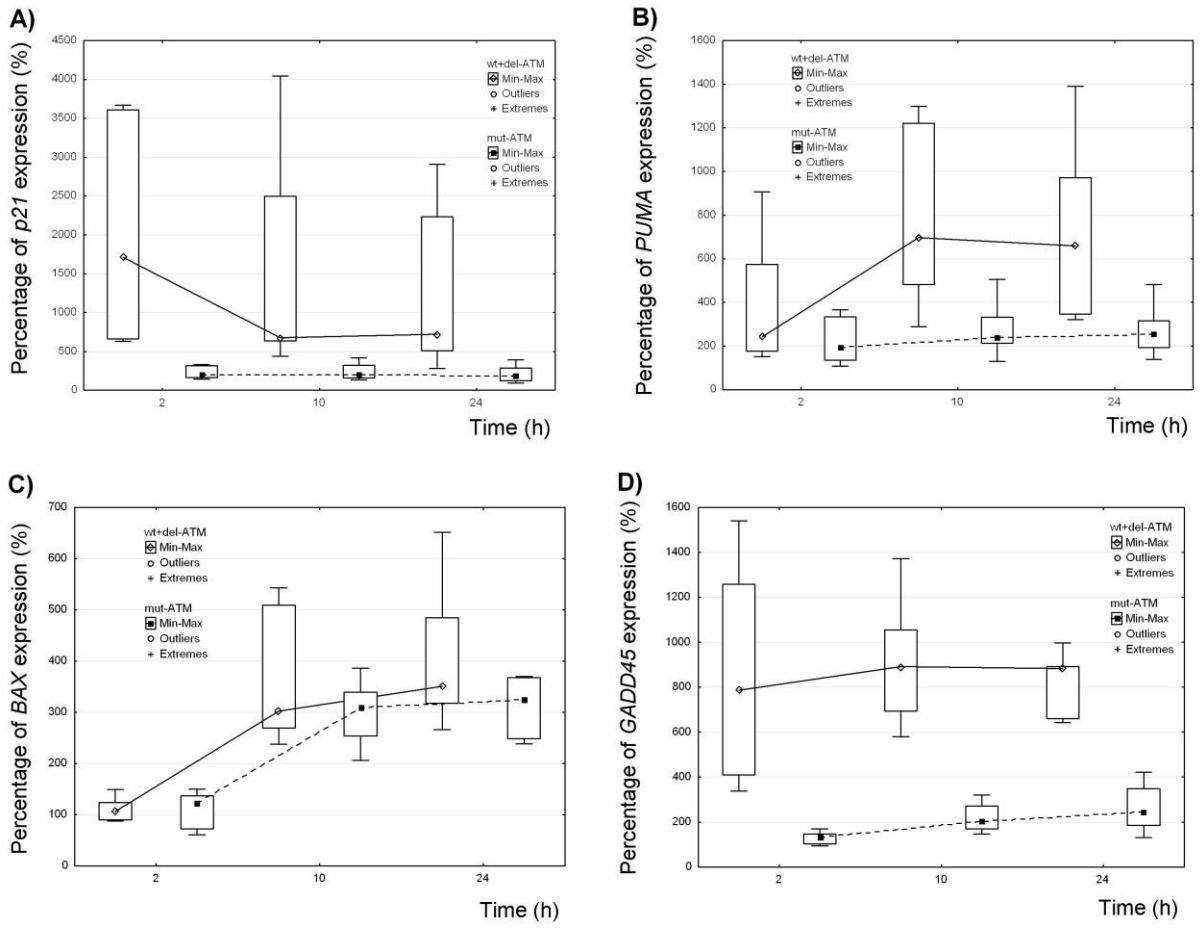
(A) after doxorubicin treatment: wt: 323%; del-ATM: 258%, NS; mut-ATM: 172%, P<0.001; def-TP53: 123%, P<0.001.
(B) after fludarabine treatment: wt: 349%; del-ATM: 329%, NS; mut-ATM: 375%, NS; def-TP53: 156%, P<0.001.



(A) after doxorubicin treatment: wt: 354%; del-ATM: 324%, NS; mut-ATM: 165%, P<0.001; def-TP53: 149%, P<0.001.

(B) after fludarabine treatment: wt: 425%; del-ATM: 500%, NS; mut-ATM: 430%, NS; def-TP53: 233%, P<0.001.

Figure S6. Activation of p53-downstream target genes after IR exposure. The p53-downstream gene (*p21, PUMA, BAX, GADD45*) expression induction after IR exposure was analyzed in *ATM*-mutated samples (mut-ATM; n=8) and in the group consisting of wt samples and cases having sole 11q- (wt+del-ATM; n=5) with preserved ATM function. *ATM*-mutated samples covered the spectrum of identified *ATM* mutations, i.e., missense mutation/11q- (n=2), missense mutation with DNE/wt (n=2), nonsense mutation/11q-, splicing mutation/UPD, splicing mutation/11q-, two short deletions. Medians of individual gene expression and the significance (Mann-Whitney U test) related to wt+del-ATM group are listed below the figures.



(A) wt+del-ATM: 1710%, 678%, 720%; mut-ATM: 207%, 206%, 183%; P=0.006, 0.004, 0.010 after 2, 10, 24h IR exposure, respectively.
(B) wt+del-ATM: 242%, 697%, 659%; mut-ATM:193%, 237%, 256%; P=0.26, 0.023, 0.010 after 2, 10, 24h IR exposure, respectively.

(C) wt+del-ATM: 106%, 303%, 351%; mut-ATM: 122%, 309%, 324%; P=1.0, 0.71, 0.34 after 2, 10, 24h IR exposure, respectively.
(D) wt+del-ATM: 786%, 891%, 883%; mut-ATM: 131%, 202%, 246%; P=0.0043, 0.0043, 0.0043 after 2, 10, 24h IR exposure, respectively.

Figure S7. Progression-free survival analysis. Analysis was limited to patients with unmutated *IGHV* and data available. Medians were 9, 16, 16.5 months in mut-ATM (n=7), del-ATM (n=11), and wt (n=16) group respectively. PFS difference for both mut-ATM and del-ATM groups related to wt patients was not significant.