Identification of molecular and functional patterns of p53 alterations in chronic lymphocytic leukemia patients in different phases of the disease

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Online Supplementary Design and Methods

Chronic lymphocytic leukemia patients

A total of 483 CLL followed at our Center were investigated at different phases of the disease: 182 patients at the time of initial diagnosis, 240 patients with progressive disease prior to treatment and 61 patients resistant to first- or second-line treatment. All patients gave their informed consent to blood collection and to the biological analyses included in the present study according to the Declaration of Helsinki. The diagnosis of CLL was based on the presence of more than 5,000 lymphocytes/μL in the peripheral blood that expressed a classic CLL immunophenotype (CD5/CD20$^+$, CD23$^+$, weak CD22$^+$, weak sIg$^+$, CD10$^-$) and morphology. IGHV gene analysis, CD38 and ZAP-70 antigen expression, and fluorescence *in situ* hybridization (FISH) analysis were performed as previously described[1-4]. At the time of mutation analysis, patients studied at diagnosis had a median age of 53 years (range 29-68) and presented a median of 20.1x10^9/L white blood cells (WBC) (range 5.79-361.7x 10^9/L). Patients with progressive disease had a median age of 59 years (range 30-92) and presented a median of 49.79x10^9/L WBC (range 1.9-511.4x10^9/L). Patients with resistant disease had a median age of 69 years (range 35-91) and presented a median of 65.08x10^9/L WBC (range 4.61-610.5x10^9/L).

DNA sequencing analysis of the TP53 gene

Mutation analysis of *TP53* exons 5 to 8 was carried out by DNA direct sequencing on an ABI PRISM 3100 automated DNA sequence analyzer (Applied Biosystems, Foster City, CA, USA), as previously described.[5] In addition, analysis of the entire coding region of *TP53* was carried out only in those cases where a discrepancy was found between p53 functional analysis and *TP53* sequencing. Mutations were validated by the IARC TP53 Mutation Database[6] (version R15, November 2010) (*http://www-p53.iarc.fr/Somatic.html*) and the UMD TP53 Mutation Database[7] (*http://p53.free.fr/Database/p53_database.html*).

Chronic lymphocytic leukemia cell culture and p53 induction

One hundred and nine CLL patients from the entire cohort 17 at diagnosis, 72 at progression and 20 with chemoresistant disease were characterized for their ability to induce p53 activation. For this purpose, CLL cells were exposed to 5Gy IR and both irradiated and non-irradiated samples were cultured for 8 and 24 h in RPMI 1640 (Cambrex BioScience Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, South Logan, UT, USA), 1% L-glutamine and 1% Pen-strepto (Euro-Clone, Milan, Italy), at 37°C in the presence of 5% CO_2. Healthy donor PBL were used as negative controls, while the *TP53*-mutated Burkitt's lymphoma B-cell line Raji, purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), as positive control.

Assessment of p53 activity by Western blot

Irradiated and non-irradiated samples after 8-h cultures were lysed in 2M Tris-HCl (pH 8), 0.5 M EDTA (pH 8), 5M sodium chloride, 0.05M sodium fluoride, 0.001M sodium orthovanadate, 10% Triton X-100 and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein extracts were separated by SDS-PAGE and blotted on nylon membranes (Immobilon-P; Millipore, Bedford, MA, USA). After blocking in 5% milk for 1 h, the membranes were probed with a monoclonal antibody to p53 (Calbiochem-Novabiochem, La Jolla, CA, USA; 1:500). A peroxidase-conjugated secondary antibody (Pierce Biotecnology, Rockford, IL, USA; 1:10000) and the ECL system (GE Healthcare, Buckinghamshire, UK) were used for detection. As a control for protein loading, the membranes were reprobed with a monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Millipore; 1:2000). Densitometric analysis was performed using the ImageJ software (NIH).

Apoptosis assay

IR-induced apoptosis was evaluated after 24-h culture using the Annexin-V technique. Briefly, CLL cells were washed in PBS and resuspended in 1xBinding buffer (10xBinding buffer: 100mM HEPES/NaOH pH 7.5, 1.4M NaCl, 25mM $CaCl_2$) adding FITC-conjugated Annexin-V and Propidium iodide (Sigma-Aldrich CO, St Louis, MS, USA) both at a final concentration of 1 μg/mL. The mixture was incubated in the dark for 10 min at room temperature and apoptotic cells were measured by flow cytometry and the data analyzed using the CellQuest software (Becton Dickinson). ΔApoptosis was calculated as:

[IR-induced apoptosis – spontaneous apoptosis].

Statistical analysis

Box plots were generated using the statistical tools available at *http://www.physics.csbsju.edu/stats/t-test_bulk_form.html*. To evaluate the significance of differences between groups, *P* values were obtained using the two-sided Student's t-test. $P \leq 0.05$ was considered statistically significant.

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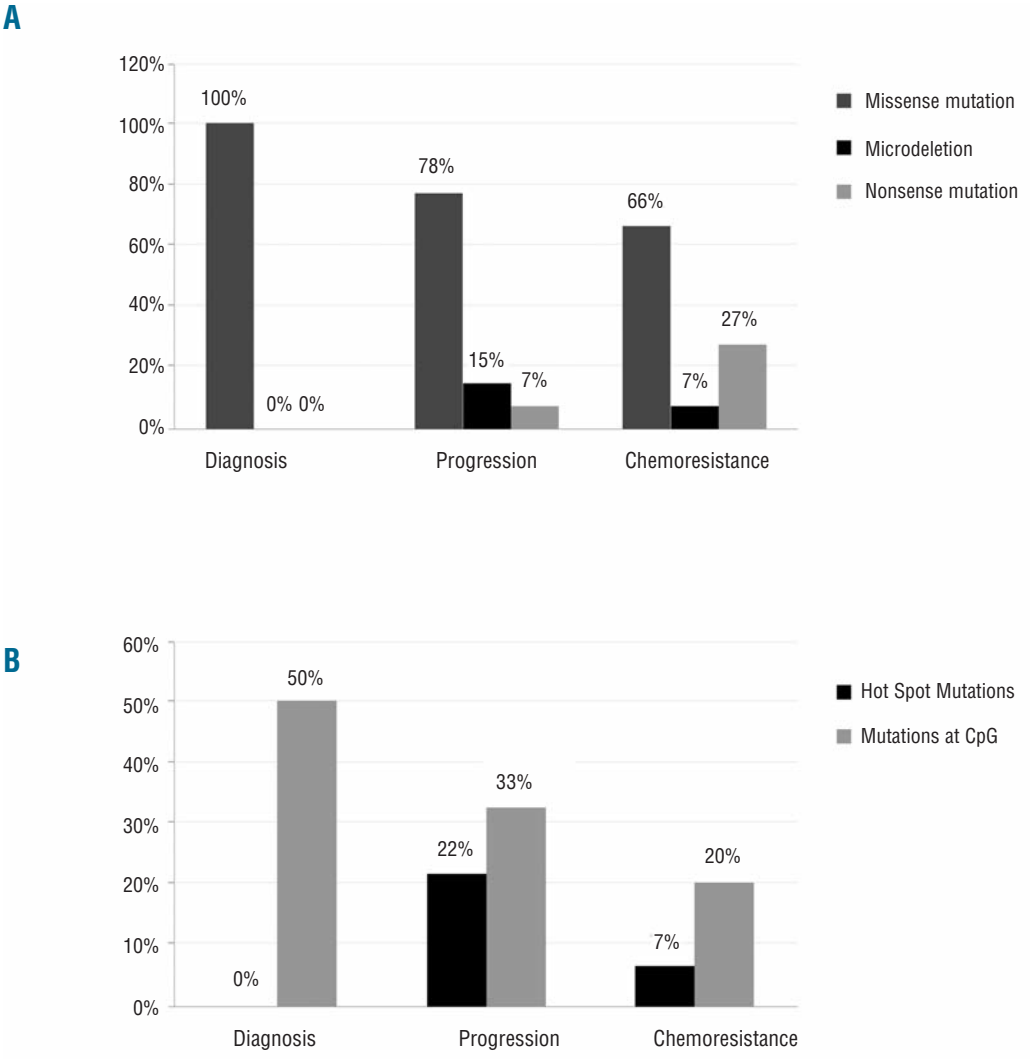

Online Supplementary Figure S1. ***TP53*** **mutation profile of CLL patients in each phase of the disease. (A) An increased proportion of cases with microdeletions (15%) was found in patients with progressive disease and a higher incidence of nonsense mutations (27%) in chemoresistant patients. In contrast, all mutations found in patients at diagnosis were missense mutations (100%). (B) Hot spot mutations were observed exclusively in progressive and chemoresistant CLL, while transitions at CpG sites were uncommon in CLL at chemoresistance.**

Online Supplementary Table S1. *TP53* mutation profile and biological characteristics of *TP53* mutated CLL patients considered either totally or divided according to their disease phase.

Mutation profile	All n/N (%)	Diagnosis n=4 (%)	Progression n=27 (%)	Chemoresistance n=15 (%) *
Mutation type				
All transitions	30/41 (73.2)	100	62.9	60
Transitions at CpG	14/30 (46.7)	50	52.9	33.3
All transversions	11/41 (26.8)	0	22.2	33.3
Transversions at CpG	2/11 (18.2)	0	33.3	0
Missense	35/41 (76.1)	100	77.8	66.6
Nonsense	6/41 (14.6)	0	7.4	26.7
Inframe/frameshift				
Deletion	5/46 (11)	0	14.8	6.7
Exons				
4	2/46 (4.3)	0	3.7	6.7
5	9/46 (19.6)	25	18.5	20
6	8/46 (17.4)	25	18.5	13.3
7	6/46 (13.1)	0	14.8	13.3
8	18/46 (39.1)	0	37.1	40
9	3/46 (6.5)	0	7.4	6.7

Biological characteristics	All n/N (%)	Diagnosis n=4 (%)	Progression n=27 (%)	Chemoresistance n=13 (%)
IGHV homology≥98%	36/44 (81.8)	50	88.9	76.9
ZAP-70 ≥20%	26/42 (61.9)	75	59.3	63.6
CD38 ≥7%	23/41 (56.1)	50	59.3	70
del17p ≥5 <20%	6/38 (15.8)	50	7.7	25
del17p ≥20%	27/38 (71.1)	25	76.9	75
del11q22-23 ≥10%	1/31 (3.2)	0	5.3	0
del13q14 ≥5%	15/31 (48.4)	75	47.4	37.5
Tris12 ≥5%	1/31 (3.2)	0	0	12.5

** 2 patients showed two mutations each.*

Online Supplementary Table S2. *TP53* mutation profile in CLL at diagnosis.

Patient	Exon	Codon	Nucleotide Change	Aminoacid Change	Transitions (Ts)/ Transversion (Tv)	CpG	Mutation type	Mutation	Mutation	Allelic status	del17p (%) by FISH
1	8	283	CGC-TGC	Arg-Cys	Ts	Yes	Missense	p.R283C	c.847C>T	Heterozygous	5
2	6	193	CAT-CGT	His-Arg	Ts	No	Missense	p.H193R	c.578A>G	Heterozygous	14
3	8	272	GTG-ATG	Val-Met	Ts	No	Missense	p.V272M	c.814G>A	Heterozygous	62
4	5	156	CGC-TGC	Arg-Cys	Ts	Yes	Missense	p.R156C	c.466C>T	Heterozygous	2

CpG: missense mutation at CpG site.

Online Supplementary Table S3. ***TP53*** **mutation profile in progressive CLL.**

Patient	Exon	Codon	Nucleotide Change	Aminoacid Change	Transitions (Ts)/ Transversion (Tv)	CpG	Mutation type	Mutation	Mutation	Allelic status	del17p (%) by FISH
1	5	184	GAT-AGC	Frameshift	–	No	Deletion	p.D184fs-X61	c.551_554del4	Heterozygous	0
2	8	282	CGG-GGG	Arg-Gly	Tv	Yes	Missense	p.R282G	c.844C>G	Homozygous	76
3	5	145	CTG-CAG	Leu-Glu	Tv	No	Missense	p.V272M	c.814G>A	Homozygous	81
4	7	248	CGG-CAG	Arg-Glu	Ts	Yes	Missense	p.R248Q	c.743G>A	Homozygous	84
5	5	179	CAT-CCT	His-Pro	Tv	No	Missense	p.H179P	c.536A>C	Heterozygous	88
6	8	283	CGC-TGC	Arg-Cys	Ts	Yes	Missense	p.R283C	c.847C>T	Homozygous	0
7	6	191	CCT-CAG	In Frame	–	No	Deletion	p.P191del	c.572_574del3	Heterozygous	87
8	8	273	CGT-CAT	Arg-His	Ts	Yes	Missense	p.R273H	c.818G>A	Heterozygous	1
9	8	273	CGT-TGT	Arg-Cys	Ts	Yes	Missense	p.R273C	c.817C>T	Heterozygous	40
10	6	209	AGA	Frameshift	–	No	Deletion	p.R209fs-X5	c.626_627del2	Heterozygous	33
11	8	275	TGT-TTT	Cys-Phe	Tv	No	Missense	p.C275F	c.824G>T	Heterozygous	9
12	7	241	TCC-TTC	Ser-Phe	Ts	No	Missense	p.S241F	c.722C>T	Homozygous	96
13	8	272	GTG-ATG	Val-Met	Ts	No	Missense	p.V272M	c.814G>A	Heterozygous	62
14	6	213	CGA-TGA	Arg-Stop	Ts	Yes	Nonsense	p.R213X	c.637C>T	Homozygous	93
15	7	248	CGG-TGG	Arg-Trp	Ts	Yes	Missense	p.R248W	c.742C>T	Heterozygous	0
16	7	245	GGC-AGC	Gly-Ser	Ts	Yes	Missense	p.G245S	c.733G>A	Heterozygous	80
17	5	175	CGC-CAC	Arg-His	Ts	Yes	Missense	p.R175H	c.524G>A	Heterozygous	nd
18	6	193	CAT-CGT	His-Arg	Ts	No	Missense	p.H193R	c.578A>G	Heterozygous	84
19	6	195	ATC-ACC	Ile-Thr	Ts	No	Missense	p.I195T	c.584T>C	Heterozygous	80
20	8	281	GAC-AAC	Asp-Asn	Ts	No	Missense	p.D281N	c.841G>A	Homozygous	94
21	8	273	CGT-CAT	Arg-His	Ts	Yes	Missense	p.R273H	c.818G>A	Homozygous	50
22	4	110	CGT-CTT	Arg-Leu	Tv	Yes	Missense	p.R110L	c.329G>T	Heterozygous	75
23	8	275	TGT-TAT	Cys-Tyr	Ts	No	Missense	p.C275Y	c.824G>A	Heterozygous	70
24	5	132	AAG-AGG	Lys-Arg	Ts	No	Missense	p.K132R	c.395A>G	Heterozygous	40
25	8	276	GCC-TGT	Frameshift	–	No	Deletion	–	c.826_829del4	Heterozygous	90
26	9	330	CTT-CCT	Leu-Pro	Ts	No	Missense	p.L330P	c.989T>C	Heterozygous	81
27	9	321	AAA-TAA	Lys-Stop	Tv	No	Nonsense	p.K321X	c.961A>T	Heterozygous	6

CpG: missense mutation at CpG site; –: not applicable; nd: not determined.

Online Supplementary Table S4. ***TP53*** **mutation profile in CLL with chemoresistant disease.**

Patient	Exon	Codon	Nucleotide Change	Aminoacid Change	Transitions (Ts)/ Transversion (Tv)	CpG	Mutation type	Mutation	Mutation	Allelic status	del17p (%) by FISH
1	7	242	TGC-TAC	Cys-Tyr	Ts	No	Missense	p.C242Y	c.725G>A	Heterozygous	30
2	5	183	TCA-TGA	Ser-Stop	Tv	No	Nonsense	p.S183X	c.548C>G	Homozygous	nd
3	8	273	CGT-CAT	Arg-His	Ts	Yes	Missense	p.R273H	c.818G>A	Heterozygous	50
4	9	321	AAA-TAA	Lys-Stop	Tv	No	Nonsense	p.K321X	c.961A>T	Homozygous	6
5	6	209	AGA	Frameshift	–	No	Deletion	p.R209fs-X5	c.626_627del2	Heterozygous	nd
6	8	272	GTG-ATG	Val-Met	Ts	No	Missense	p.V272M	c.814G>A	Heterozygous	nd
6	7	235	AAC-GAC	Asn-Asp	Ts	No	Missense	p.N235D	c.703A>G	Heterozygous	nd
7	6	213	CGA-TGA	Arg-Stop	Ts	Yes	Nonsense	p.R213X	c.637C>T	Homozygous	nd
8	8	278	CCT-CTT	Pro-Leu	Ts	No	Missense	p.P278L	c.833C>T	Homozygous	nd
9	8	278	CCT-CGT	Pro-Arg	Tv	No	Missense	p.P278R	c.833C>G	Heterozygous	87
10	5	126	TAC-TAG	Tyr- Stop	Tv	No	Nonsense	p.Y126X	c.378C>G	Heterozygous	95
11	5	152	CCG-CTG	Pro-Leu	Ts	Yes	Missense	p.P152L	c.455C>T	Homozygous	95
12	4	113	TTC-TCC	Phe-Ser	Ts	No	Missense	p.F113L	c.338T>C	Heterozygous	0
13	8	274	GTT-GAT	Val-Asp	Tv	No	Missense	p.V274D	c.821T>A	Heterozygous	50
13	8	278	CCT-CTT	Pro-Leu	Ts	No	Missense	p.P278S	c.833C>T	Heterozygous	50

CpG: missense mutation at CpG site; –: not applicable; nd: not determined.

Online Supplementary Table S5. Correlation between p53 dysfunctions and *TP53* mutation profile in CLL patients.

Phase of the disease	Patient	p53 dysfunction type	Exon	Codon	Nucleotide Change	Aminoacid Change	Transition (Ts)/ Transversion (Tv)	Mutation type	Allelic status	del17p (%) by FISH
Diagnosis (n=1)	2	II	6	193	CAT-CGT	His-Arg	Ts	Missense	Heterozygous	14
	2	II	8	282	CGG-GGG	Arg-Gly	Tv	Missense	Homozygous	76
	6	I	8	283	CGC-TGC	Arg-Cys	Ts	Missense	Homozygous	0
	7	I	6	191	CCT-CAG	In Frame	–	Deletion	Heterozygous	87
	8	I	8	273	CGT-CAT	Arg-His	Ts	Missense	Heterozygous	1
	11	I	8	275	TGT-TTT	Cys-Phe	Tv	Missense	Heterozygous	9
	12	II	7	241	TCC-TTC	Ser-Phe	Ts	Missense	Homozygous	96
Progression (n=17)	14	III	6	213	CGA-TGA	Arg-Stop	Ts	Nonsense	Homozygous	93
	15	I	7	248	CGG-TGG	Arg-Trp	Ts	Missense	Heterozygous	0
	17	I	5	175	CGC-CAC	Arg-His	Ts	Missense	Heterozygous	nd
	18	II	6	193	CAT-CGT	His-Arg	Ts	Missense	Heterozygous	84
	19	I	6	195	ATC-ACC	Ile-Thr	Ts	Missense	Heterozygous	80
	20	II	8	281	GAC-AAC	Asp-Asn	Ts	Missense	Homozygous	94
	22	II	4	110	CGT-CTT	Arg-Leu	Tv	Missense	Heterozygous	75
	23	I	8	275	TGT-TAT	Cys-Tyr	Ts	Missense	Heterozygous	70
	25	III	8	276	GCC-TGT	Frameshift	–	Deletion	Heterozygous	90
	26	I	9	330	CTT-CCT	Leu-Pro	Ts	Missense	Heterozygous	81
	27	II	9	321	AAA-TAA	Lys-Stop	Tv	Nonsense	Heterozygous	6
	3	I	8	273	CGT-CAT	Arg-His	Ts	Missense	Heterozygous	50
	4	II	9	321	AAA-TAA	Lys-Stop	Tv	Nonsense	Homozygous	6
Resistance (n=6)	6	II	8	272	GTG-ATG	Val-Met	Ts	Missense	Heterozygous	nd
	6	II	7	235	AAC-GAC	Asn-Asp	Ts	Missense	Heterozygous	nd
	9	I	8	278	CCT-CGT	Pro-Arg	Tv	Missense	Heterozygous	87
	10	II	5	126	TAC-TAG	Tyr- Stop	Tv	Nonsense	Heterozygous	95
	12	II	4	113	TTC-TCC	Phe-Ser	Ts	Missense	Heterozygous	5

–: not applicable; nd: not determined.

Online Supplementary Table S6. Biological characteristics of p53 dysfunctional CLL patients according to the type of p53 dysfunction.

Biological characteristics	All dysfunctions (%)	Type I (%)	Type II (%)	Type III (%)
IGHV homology≥98%	78.6	71.4	90.9	100
ZAP-70 ≥20	65.2	58.3	77.8	50
CD38 ≥7%	56.5	36.4	80	50
del17p ≥5 <20%	20	8.3	36.4	0
del17p ≥20%	60	50	63.6	100
del11q22-23 ≥10%	4.2	0	9.1	0
del13q14 ≥5%	33.3	45.5	18.2	50
Tris12 ≥5%	8.3	18.2	0	0