Integration of B-cell receptor-induced ERK1/2 phosphorylation and mutations of *SF3B1* gene refines prognosis in treatment-naïve chronic lymphocytic leukemia

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SUPPLEMENTARY DATA

METHODS

Single Cell Network Profiling (SCNP) assay and metric. PBMCs were analyzed by SCNP on a FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, NJ, US), as previously described.[1] Fixed and permeabilized cells were stained with cocktails of fluorochrome-conjugated antibodies: CD3-APCCy7 (clone UCHT1; BioLegend, San Diego, CA, US), CD5-V450 (clone L17F12; BD Biosciences, San Jose, CA, US), CD14-APCCy7 (clone M5E2; BioLegend), CD19-PE (clone SJ25C1; eBioscience, San Diego, CA, US), cPARP-AlexaFluor647 (clone F21-852; BD Biosciences), p-ERK-AlexaFluor488 (Thr202/Tyr204; clone D13.14.4E; Cell Signalling Technology, Danvers, MA, US). Dead cells and debris were excluded on the basis of forward scatter, side scatter, and cPARP signals. All analyses were gated on CLL cells, which were identified as CD3/CD14 negative cells co-expressing CD5 and CD19. The raw instrument fluorescence intensities were converted into calibrated intensity metrics (equivalent number of reference fluorophores, ERF).[1] The anti-IgM-induced ERK1/2 phosphorylation (anti-IgM→pERK1/2) was measured using the Uu metric, the Mann-Whitney U statistic comparing the ERF values of the modulated and unmodulated wells. The pre-specified anti-IgM→p-ERK1/2 Uu cut-off of 0.66 was used to separate patients into groups, based on our previous validation study.[1]

Genetic analysis. Cytogenetic abnormalities were evaluated by fluorescence *in situ* hybridization (FISH), according to the hierarchical risk model of FISH anomalies.[2] The mutation hot spots of the *NOTCH1*, *SF3B1*, *TP53*, *BIRC3, and MYD88* genes were analyzed by PCR amplification and direct sequencing of high-molecular-weight genomic DNA, as previously described.[3]

NOTCH1 mutations were detected in 17/146 (12%) cell samples. Thirteen patients of 17 (76%) carried the highly recurrent c.7544_7545delCT deletion, 1/17 (6%) the c.7462C>T mutation and 1/17 (6%) c.7593_7594insC mutation; 2/17 (12%) lesions were non-sense events. *SF3B1* genetic lesions were carried by cells from 19/146 (13%) patients. Missense mutations in the *SF3B1* hotspots affected codons 625 (p.R625L, n=1), 626 (p.N626I and p.N626Y, n=2), 700 (p.K700E, n=10), 704 (p.I704F, n=1), 740 (p.G740E, n=2), 742 (p.G742D, n=1), 760 (p.E760Q, n=1), 765 (p.Y765C, n=1). *TP53* gene mutations were detected in 11/146 (8%) cell samples, with 1 patient harboring 2 mutations.

Statistical analysis. Clinical and biological features between patients were compared with Fisher's exact test, χ^2 test or Student's t-test, as appropriate. Two-sample Wilcoxon signed-rank sum test was used to compare ERK1/2 phosphorylation in unmodulated and anti-IgM-modulated samples. Mann-Whitney test was used to compare phosphorylation of ERK1/2 in patients grouped on the basis of standard prognostic factors (*IGHV* mutational status and CD38 expression). Kruskal-Wallis test was used to compare phosphorylation of ERK1/2 in patients grouped on the basis of cytogenetic alterations [*del(13q), trisomy 12, del(11q), del(17p)*].

Time-to-first-treatment (TTFT) was calculated from the date of diagnosis to the date of initial therapy. Patients who did not receive any treatment during follow-up were censored at their last follow-up date. TTFT curves estimated using the Kaplan-Meier method for the respective groups of patients were compared using the log-rank χ^2 test. Univariate and bivariate models for TTFT were generated using Cox proportional hazards regression. Differences between

data were considered statistically significant for *P* values ≤0.05. Graphing and statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, US) or StatView (Abacus Concepts, Berkeley, CA, US).

References.

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Supplementary Table S1. Characteristics of CLL patients[1]

	Entire patient set (n=152)	**Binet stage A patients (n=112)**	**P value Entire set *vs* Binet A**
Age at the diagnosis			
median (range), years	66 (28 to 90)	66 (36 to 89)	NS
Gender			
male	102 (67%)	76 (68%)	NS
female	50 (33%)	36 (32%)	
Stage			
Binet A	112 (74%)	112 (100%)	NA
Binet B	30 (20%)	-	
Binet C	8 (5%)	-	
unavailable	2 (1%)	-	
IGHV[2]			
mutated	75 (49%)	58 (52%)	NS
unmutated	59 (39%)	39 (35%)	
unavailable	18 (12%)	15 (13%)	
CD38[3]			
negative	112 (74%)	87 (78%)	NS
positive	40 (26%)	25 (22%)	
Cytogenetic			
del(13q)	66 (43%)	51 (45%)	NS
trisomy 12	18 (12%)	13 (12%)	
del(11q)	14 (9%)	8 (7%)	
del(17p)	8 (5%)	7 (6%)	
normal	42 (28%)	30 (27%)	
unavailable	11 (7%)	8 (7%)	
Follow up			
median (range), months	50 (0 to 120)	50 (0 to 120)	NS
Disease progression, requiring treatment during follow up	63 (41%)	33 (29%)	NA
unavailable	4 (3%)	2 (2%)	
TTFT			
median (range), months	16 (0 to 120)	25 (0 to 120)	NS

[1]Patients were enrolled from January 2006 to December 2013 in a regional prospective registry (CLL Veneto project) at the Hematology Units of Verona (Azienda Ospedaliera Universitaria Integrata), Vicenza (San Bortolo Hospital), and Padua (Azienda Ospedaliera).
[2]*IGHV* sequencing utilized a 2% cut-off to discriminate unmutated (UM) from mutated (M) *IGHV*.
[3]CD38 was determined using a 30% cut-off.
NS: not significant
NA: not applicable

Supplementary Table S2. Comparison of early versus advanced clinical stage, and treated versus untreated patients

	Early *vs* advanced clinical stage			**Untreated *vs* treated patients**		
	Binet A n, (%)	**Binet B-C n, (%)**	***P* value**	**Untreated n, (%)**	**Treated n, (%)**	***P* value**
Anti-IgM→p-ERK >0.66 Uu	66/112, (58%)	25/38, (66%)	NS	41/85, (48%)	49/63, (78%)	**0.0003**
IGHV unmutated	39/97, (40%)	19/36, (53%)	NS	19/73, (26%)	39/60, (65%)	**< 0.0001**
CD38 positive	25/112, (22%)	14/38, (37%)	NS	13/85, (15%)	26/63, (41%)	**0.0006**
NOTCH1 mutated	10/107, (9%)	4/37, (11%)	NS	7/81, (9%)	7/61, (11%)	NS
SF3B1 mutated	8/107, (7%)	11/37, (30%)	**0.0014**	4/81, (5%)	15/61, (24%)	**0.0009**
TP53 mutated	10/107, (9%)	1/37, (3%)	NS	3/81, (4%)	7/61, (11%)	NS
Del(13q)	51/104, (49%)	15/37, (40%)	NS	44/79, (56%)	22/60, (37%)	**0.0392**
Trisomy 12	13/104, (12%)	5/37, (13%)	NS	8/79, (10%)	10/60, (17%)	NS
Del(11q)	8/104, (8%)	6/37, (16%)	NS	4/79, (5%)	11/60, (18%)	**0.0244**
Del(17p)	7/104, (7%)	1/37, (3%)	NS	2/79, (2%)	5/60, (8%)	NS

NS: not significant

Supplementary Table S3. Gene mutation distribution in selected CLL subset

	NOTCH1			***SF3B1***			***TP53***			***TP53 + del(17p)***		
CLL subset	**wt n, (%)**	**mut. n, (%)**	***P* value**	**wt n, (%)**	**mut. n, (%)**	***P* value**	**wt n, (%)**	**mut. n, (%)**	***P* value**	**wt-*TP53* and not *del(17p)* n, (%)**	**m-*TP53* and/or *del(17p)* n, (%)**	***P* value**
Anti-IgM→p-ERK1/2 >0.66 Uu	73/131, (56%)	11/15, (73%)	NS	73/127, (57%)	17/19, (89%)	**0.0098**	80/135, (59%)	10/11, (91%)	**0.0514**	72/121, (59%)	11/14, (79%)	NS
IGHV unmutated	51/119, (43%)	8/11, (73%)	NS	43/111, (39%)	16/19, (84%)	**0.0003**	52/121, (43%)	7/9, (78%)	NS	-	-	NA
CD38 positive	32/131, (24%)	8/15, (53%)	**0.0291**	32/127, (25%)	8/19, (42%)	NS	34/135, (35%)	6/11, (54%)	NS	-	-	NA
Del(13q)	52/122, (43%)	5/13, (38%)	NS	54/116, (46.5%)	7/19, (37%)	NS	59/126, (47%)	2/9, (22%)	NS	-	-	NA
Trisomy 12	13/122, (11%)	4/13, (31%)	**0.0606**	16/116, (14%)	1/19, (5%)	NS	15/126, (12%)	2/9, (22%)	NS	-	-	NA
Del(11q)	13/122, (11%)	1/13, (8%)	NS	11/116, (9%)	0/19, (0%)	NS	12/126, (10%)	2/9, (22%)	NS	-	-	NA
Del(17p)	7/122, (6%)	1/13, (8%)	NS	8/116, (7%)	0/19, (0%)	NS	5/126, (4%)	3/9, (33%)	**0.0099**	-	-	NA

NS: not significant; NA: not applicable

Supplementary Table S4A. Selected Cox proportional hazards models for TTFT in the entire patient set (n=125)

Variable 1	**HR (CI 95%)**[1]	***P* value var1**	**Variable 2**	**HR (CI 95%)**[1]	***P* value var2**
Anti-IgM→p-ERK1/2	1.296 (0.643-2.614)	0.46	NA	NA	NA
IGHV unmutated	2.785 (1.428-5.434)	**0.002**	NA	NA	NA
CD38 positive	1.245 (0.642-2.552)	0.56	NA	NA	NA
SF3B1 mutated	2.304 (1.204-4.405)	**0.01**	NA	NA	NA
Anti-IgM→p-ERK1/2	1.653 (0.854-3.198)	0.13	*IGHV* unmutated	3.300 (1.855-5.847)	**< 0.0001**
Anti-IgM→p-ERK1/2	1.922 (1.033-3.566)	**0.03**	*SF3B1* mutated	3.731 (2.016-6.896)	**0.0001**

[1]HR: Hazard Ratio; CI: Confidence Interval

Supplementary Table S4B. Selected Cox proportional hazards models for TTFT in Binet stage A patients (n=90)

Variable 1	**HR (CI 95%)**[1]	***P* value var1**	**Variable 2**	**HR (CI 95%)**[1]	***P* value var2**
Anti-IgM→p-ERK1/2	1.071 (0.334-2.587)	0.89	NA	NA	NA
IGHV unmutated	5.617 (1.538-15.384)	**0.0008**	NA	NA	NA
CD38 positive	1.683 (0.547-2.452)	0.19	NA	NA	NA
TP53 mutated	2.145 (0.833-5.556	0.11	NA	NA	NA
SF3B1 mutated	3.355 (1.230-9.174)	**0.01**	NA	NA	NA
Anti-IgM→p-ERK1/2	1.523 (0.605-3.837)	0.37	*IGHV* unmutated	6.578 (2.724-15.873)	**< 0.0001**
Anti-IgM→p-ERK1/2	2.437 (0.976-6.089)	**0.05**	*SF3B1* mutated	5.319 (2.053-13.698)	**0.0006**

[1]HR: Hazard Ratio; CI: Confidence Interval