Chronic lymphocytic leukemia cells in lymph nodes show frequent NOTCH1 activation

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Supplemental Information.

MATERIALS AND METHODS

Patients and samples

A total of 155 lymph node samples from 147 patients diagnosed with CLL according to the World Health Organization Criteria were included in the study. 127 samples were taken at the time of diagnosis and 25 at progression. Lymph node biopsies were routinely performed in CLL patients with massive lymphadenopathies or sudden growth of the lymph nodes. The samples correspond to consecutive samples, collected by different centers, where the CLL diagnosis has been clearly established and there was enough paraffin embedded material for sequencing and for immunohistochemical studies. Three samples yielded no data. Clinical and biological data at the time of diagnosis, treatment and follow-up were collected from 108 patients. Patients' characteristics are summarized in **Table 1**. The study, the patient information sheet and the informed consent form were approved by the Ethics Committees of the HU Puerta de Hierro-Majadahonda and HU Marqués de Valdecilla.

NOTCH1 and SF3B1 mutations detection

We extracted DNA from tumoral formalin-fixed, paraffin-embedded (FFPE) samples using a QIAamp® DNA FFPE Tissue kit (Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturer's protocol.

For the NOTCH1 mutation c.7544_7545delCT (p.P2515fs*4) screenings, qBiomarker Somatic Mutation Assay SMPH009713A by SabBioscence-Quiagen, were used. This is a semi-quantitative method that has sensitivity enough to detect mutations present in as low as 5% of mutated alleles in fresh or frozen samples and 10% in DNA obtained from FFPE material. In some selected cases, the presence of the mutations was also validated by capillary sequencing:

NOTCH1_2515_FW: TACTTGAAGGCCTCCGGAAT

NOTCH1_2515_RV: CTCGCAGCACAGCTACTCCT

Two approaches were used to detect mutations in SF3B1. The c.2098A>G (p.K700E) mutation was detected by qBiomarker Mutation Assay SMPH032120A, and exons 14 and 16

were analyzed by PCR amplification and capillary sequencing by Sanger method using the following primers:

SF3B1_742_FW: TCTTCATTAAAGTTAAGGCGACA

SF3B1_742_RV: TTCCTCATCAGGAGACTGGAA

SF3B1_EX15_FW: TGCAGTTTGGCTGAATAGTTG

SF3B1_EX15_RV: CCAATAGCCTTCAAGAAAGCAG

SF3B1_EX15.2-FW: CCTTCAAGAAAGCAGCCAAA

SF3B1-EX15.2-RV: TTGGCTGAAGCAGCAACTC

SF3B1_EX14A_FW: GAGTCCAGTCTGGGCAACAT

SF3B1_EX14A_RV: CCCTGGGCATTCCTTCTTTA

SF3B1_EX14B_FW: TGTTGTACAATCTTAATACCAGTGTG

SF3B1_EX14B_RV: CCAACTCATGACTGTCCTTTCTT

Tissue microarray construction and immunohistochemical analysis

Three tissue microarrays (TMAs) were constructed from 150 samples, using six reactive tonsillectomy and lymph node specimens as controls. Representative areas from FFPE samples were carefully selected on H&E-stained sections and two cores of 1 mm diameter were obtained from each specimen. The tissue cores were precisely arrayed into a new paraffin block using a TMA workstation (Beecher Instruments, Silver Spring, MD) (23). Immunohistochemical (IHC) expression was assessed by routine IHC techniques using the following antibodies: NOTCH1 (Rabbit mAb (D3B8), Cell Signaling); Ki67 (clone MIB1, Dako); NFATc1 (clone 7A6, BD Biosciences); p50 (clone E381, Millipore); p52 (Millipore); c-MYC (clone Y29, Epitomics); MUM1 (clone MUM1p, Dako); XBP1s (McAb, CNIO); HES-1 (Rabbit mAb (D6P2U), Cell Signaling); LEF1 (clone EPR2029Y, Abcam), JAG1 (clone EPR4290, Abcam).

FISH in TMAs

FISH analyses were performed on 3-µm TMA tissue sections using commercial probes: DNA-FISH LSI MYC Dual Color Break Apart (01N63-020; Abbott Molecular); Vysis LSI p53 / LSI ATM and LSI D13S319 / LSI 13q34 / CEP 12 Multi-color Probe (08L53-020; Abbott Molecular); DNA-FISH LSI TP53 SpectrumOrange/CEP 17 SpectrumGreen (05N56-020; Abbott Molecular), DNA-FISH LSI IGH Dual Color Break Apart (KBI-10601; Kreactech) following standard procedures. 10 µl of a prediluted probe were applied to the specimen. At least 100 intact, non-overlapping nuclei were analyzed on each TMA core. Discordant duplicates were reevaluated by two observers (AB, SG). Control values were previously established based on the mean plus

three standard deviations of 10 control samples. Nuclei were scored as rearranged if at least one split orange-green signal was observed. Gains were reported when three or more fusion signals were observed. The cut-off value for chromosome gain or rearrangement was 15% in both cases. Heterozygous deletions were defined as > 50% nuclei containing one signal of locus probe and two signals of the reference probe.

Statistics

Statistical analyses were done with SPSS (version 19.0.0) (IBM Corporation, Armonk, NY, USA). Dichotomous variables were compared using the chi-square test. The log-rank test was used to examine overall survival (OS) and time to treatment (TTT) with various parameters as covariates. All reported p-values are two-sided and were considered significant if less than 0.05. OS was measured from the date of diagnosis until last follow-up or death by CLL. TTT was calculated as the period between diagnosis and initial treatment.

Table S1. Statistical analysis of clinical and phenotypical parameters at diagnosis *versus* mutational status of *NOTCH1* and *SF3B1* genes in the cohort of 108 patients with CLL.

		NOTCH1 wt N (%)	NOTCH1 mut N (%)	CHI2 P-value	SF3B1 wt N (%)	SF3B1 mut N (%)	CHI2 P-value
Gender	Male	47 (62.7%)	11 (47.8%)	0.400	52 (58.4%)	10 (76.9%)	0.147
	Female	25 (33.3%)	9 (39.1%)		32 (36.0%)	2 (15.4%)	
	ND	3 (4.0%)	3 (13.0%)		5 (5.6%)	1 (7.7%)	
Age > 65y	no	28 (37.3%)	8 (34.8%)	0.905	31 (34.8%)	6 (46.2%)	0.459
	yes	41 (54.7%)	11 (47.8%)		49 (55.1%)	6 (46.2%)	
	ND	6 (8.0%)	4 (17.4%)		9 (10.1%)	1 (7.7%)	
Second tumors	no	64 (85.3%)	21 (91.3%)	0.540	78 (87.6%)	10 (76.9%)	0.676
	yes	10 (13.3%)	2 (8.7%)		11 (12.4%)	2 (15.4%)	
	ND	1 (1.3%)	0 (0.0%)		0 (0.0%)	1 (7.7%)	
Advanced stage	no	35 (46.7%)	10 (43.5%)	0.918	40 (44.9%)	7 (53.8%)	0.546
	yes	15 (20.0%)	4 (17.4%)		19 (21.3%)	2 (15.4%)	
	ND	25 (33.3%)	9 (39.1%)		30 (33.7%)	4 (30.8%)	
Need of treatment	NO	30 (40.0%)	9 (39.1%)	0.941	33 (37.1%)	6 (46.2%)	0.529
	yes	45 (60.0%)	14 (60.9%)		56 (62.9%)	7 (53.8%)	
Exitus	No	34 (45.3%)	10 (43.5%)	0.795	39 (43.8%)	4 (30.8%)	0.340
	yes	39 (52.0%)	13 (56.5%)		48 (53.9%)	9 (69.2%)	
	ND	2 (2.7%)	0 (0.0%)		2 (2.2%)	0 (0.0%)	
Death by disease	No	43 (57.3%)	12 (52.2%)	0.569	49 (55.1%)	6 (46.2%)	0.492
	yes	30 (40.0%)	11 (47.8%)		38 (42.7%)	7 (53.8%)	
	ND	2 (2.7%)	0 (0.0%)		2 (2.2%)	0 (0.0%)	
13q14d13	no	16 (21.3%)	5 (21.7%)	0.185	16 (18.0%)	5 (38.5%)	0.225
	yes	6 (8.0%)	0 (0.0%)		5 (5.6%)	0 (0.0%)	
	ND	53 (70.7%)	18 (78.3%)		68 (76.4%)	8 (61.5%)	
11q23	no	41 (54.7%)	10 (43.5%)	0.863	43 (48.3%)	8 (61.5%)	0.295
	yes	5 (6.7%)	1 (4.3%)		6 (6.7%)	0 (0.0%)	
	ND	29 (38.7%)	12 (52.2%)		40 (44.9%)	5 (38.5%)	
tri12	no	15 (20.0%)	2 (8.7%)	0.184	12 (13.5%)	4 (30.8%)	0.405
	yes	6 (8.0%)	3 (13.0%)		8 (9.0%)	1 (7.7%)	
	ND	54 (72.0%)	18 (78.3%)		69 (77.5%)	8 (61.5%)	
17p13	no	38 (50.7%)	14 (60.9%)	0.231	46 (51.7%)	7 (53.8%)	0.513
	yes	4 (5.3%)	0 (0.0%)		3 (3.4%)	1 (7.7%)	
	ND	33 (44.0%)	9 (39.1%)		40 (44.9%)	5 (38.5%)	
Mutated IgHv	no	28 (37.3%)	10 (43.5%)	0.193	34 (38.2%)	6 (46.2%)	0.781
	yes	21 (28.0%)	3 (13.0%)		21 (23.6%)	3 (23.1%)	
	ND	26 (34.7%)	10 (43.5%)		34 (38.2%)	4 (30.8%)	
ZAP70	no	17 (22.7%)	2 (8.7%)	0.181	16 (18.0%)	3 (23.1%)	0.732
	yes	13 (17.3%)	5 (21.7%)		16 (18.0%)	4 (30.8%)	
	ND	45 (60.0%)	16 (69.6%)		57 (64.0%)	6 (46.2%)	
CD38	no	13 (17.3%)	0 (0.0%)	0.043	12 (13.5%)	2 (15.4%)	0.699
	yes	26 (44.7%)	9 (39.1%)		30 (33.8%)	7 (53.9%)	
	ND	36 (48.0%)	14 (60.9%)		47 (52.8%)	4 (30.8%)	
β2-microglobuline	no	16 (21.3%)	3 (13.0%)	0.669	16 (18.0%)	4 (30.8%)	0.238
	yes	27 (36.0%)	7 (30.4%)		31 (34.8%)	3 (23.1%)	
	ND	32 (42.7%)	13 (56.5%)		42 (47.2%)	6 (46.2%)	
LDH	no	46 (61.3%)	9 (39.1%)	0.045	52 (58.4%)	8 (61.5%)	0.112
	yes	11 (14.7%)	7 (30.4%)		17 (19.1%)	0 (0.0%)	
	ND	18 (24.0%)	7 (30.4%)		20 (22.5%)	5 (38.5%)	
Lymphocytosis	no	51 (68.0%)	12 (52.2%)	0.958	57 (64.0%)	8 (61.5%)	0.157
	yes	4 (5.3%)	1 (4.3%)		4 (4.5%)	2 (15.4%)	
	ND	20 (26.7%)	10 (43.5%)		28 (31.5%)	3 (23.1%)	

N: number of cases; wt: wild type; mut: mutated; y: years; tri: trisomy; LDH: lactate dehydrogenase; ND: no data

Table S2. Statistical analysis of expression of NOTCH1 and NOTCH1 targets *versus NOTCH1* mutational status genes in the cohort of lnCLL samples.

		Notch1 wt		Notch1 mut		
	N total	**N**	**%**	**N**	**(%)**	**P-value**
NOTCH1	**120**					0.126
	neg	9	9.9%	6	20.7%	
	nuc	82	90.1%	23	79.3%	
NFAT	**128**					**0.004**
	cit	94	79.7%	4	40.00%	
	nuc	24	20.3%	6	60.00%	
P52	**128**					**0.016**
	cit	90	95.7%	23	82.1%	
	nuc	4	4.3%	5	17.9%	
P50	**120**					0.159
	cit	79	86.8%	22	75.9%	
	nuc	12	13.2%	7	24.1%	
CMYC	**117**					**0.043**
	neg	33	36.3%	4	15.4%	
	pos	58	63.7%	22	84.6%	
MUM1	**116**					0.384
	neg	21	23.9%	9	32.1%	
	pos	67	76.1%	19	67.9%	
XBP1	**125**					0.29
	neg	55	56.7%	19	67.9%	
	pos	42	43.3%	9	32.1%	
LEF1	**118**					0.78
	neg	33	36.3%	9	33.3%	
	pos	58	63.7%	18	66.7%	
HES1	**118**					0.544
	neg	80	88.9%	26	92.9%	
	pos	10	11.1%	2	7.1%	
JAG1	**95**					0.684
	neg	45	67.2%	20	71.4%	
	pos	22	32.8%	8	28.6%	
Ki67	**120**					0.512
	neg	47	51.6%	17	58.6%	
	pos	44	48.4%	12	41.4%	

N: number of cases; wt: wild type; mut: mutated; cit: cytoplasmic expression; nuc: nuclear expression; neg: negative expression; pos: positive expression.

Table S3. Statistical analysis of expression of NOTCH1 targets *versus* NOTCH1 expression in the cohort of lnCLL samples.

		Notch1 neg		Notch1 nuc		
	N total	**N**	**%**	**N**	**(%)**	**P-value**
NFAT	**129**					0.776
	cit	13	76.5%	82	73.2%	
	nuc	4	23.5%	30	26.8%	
P52	**123**					0.241
	cit	17	100.0%	98	92.5%	
	nuc	0	0.0%	8	7.5%	
P50	**120**					0.558
	cit	15	88.2%	85	82.5%	
	nuc	2	11.8%	18	17.5%	
CMYC	**119**					0.662
	neg	5	33.3%	29	27.9%	
	pos	10	66.7%	75	72.1%	
MUM1	**116**					0.145
	neg	2	13.3%	32	31.7%	
	pos	13	86.7%	69	68.3%	
XBP1	**127**					0.437
	neg	11	68.8%	65	58.6%	
	pos	5	31.3%	46	41.4%	
LEF1	**123**					0.79
	neg	6	35.3%	41	38.7%	
	pos	11	64.7%	65	61.3%	
HES1	**117**					0.591
	neg	16	94.1%	90	90.0%	
	pos	1	5.9%	10	10.0%	
Ki67	**123**					0.752
	neg	10	58.8%	58	54.7%	
	pos	7	41.2%	48	45.3%	

N: number of cases; wt: wild type; mut: mutated; cit: cytoplasmic expression; nuc: nuclear expression; neg: negative expression; pos: positive expression.

Table S4. Statistical analysis of expression of NOTCH1 and NOTCH1 targets *versus* JAG1 expression in the cohort of lnCLL samples.

		JAG1 neg		JAG1 pos		
	N total	**N**	**%**	**N**	**(%)**	**P-value**
NOTCH1	**100**					0.687
	neg	9	12.9%	3	10.0%	
	nuc	61	87.1%	27	90.0%	
P52	**97**					0.349
	cit	62	91.2%	28	96.6%	
	nuc	6	8.8%	1	3.4%	
P50	**93**					0.618
	cit	57	83.8%	22	88.0%	
	nuc	11	16.2%	3	12.0%	
CMYC	**91**					**0.023**
	neg	22	34.4%	3	11.1%	
	pos	42	65.6%	24	88.9%	
MUM1	**90**					**0.021**
	neg	24	38.7%	4	14.3%	
	pos	38	61.3%	24	85.7%	
XBP1	**99**					0.396
	neg	45	64.3%	16	55.2%	
	pos	25	35.7%	13	44.8%	
LEF1	**93**					**0.008**
	neg	32	50.0%	6	20.7%	
	pos	32	50.0%	23	79.3%	
HES1	**94**					0.194
	neg	59	92.2%	25	83.3%	
	pos	5	7.8%	5	16.7%	
NFAT1	**104**					0.599
	cit	58	77.3%	21	72.4%	
	nuc	17	22.7%	8	27.6%	

N: number of cases; wt: wild type; mut: mutated; cit: cytoplasmic expression; nuc: nuclear expression; neg: negative expression; pos: positive expression.