Low frequency mutations independently predict poor treatment-free survival in early stage chronic lymphocytic leukemia and monoclonal B-cell lymphocytosis

Recent studies employing next generation sequencing (NGS) technologies have identified novel recurring mutations in monoclonal B-cell lymphocytosis (cMBL) and chronic lymphocytic leukemia (CLL).[1] *NOTCH1* and *SF3B1* mutations are the most prevalent and are associated with reduced survival independent of established clinical and biological variables. However, we have limited understanding of the impact on clinical outcome of less prevalent mutations, especially when identified at diagnosis in patients with cMBL or Binet stage A CLL. To address this question, we have investigated the clinical significance of *SF3B1, NOTCH1* and four 'low frequency' mutations: *POT1, XPO1, MYD88, BIRC3* in a single center cohort of well-characterized patients. A fifth gene (*FBXW7*) was screened only in cases with trisomy 12, in view of the known strong association between the two abnormalities.[2]

This study included 206 previously untreated patients with a diagnosis of either Binet stage A CLL [n=116] or cMBL [n=-90] (*Online Supplementary Table S1*) according to the National Cancer Institute-Working Group (NCI-WG) criteria up-dated in 2008.[3] Follow up ranged from one to 35 years with a median of ten years. One hundred and ten patients (53%) remained in stable Binet Stage A or cMBL while 29 of 90 (32%) of patients with cMBL evolved to Binet stage A during the observational period. Sixty-seven (33%) patients progressed to Binet stage B or C disease and required treatment. Of these, 21 (23%) progressed from cMBL and 46 (40%) from Binet stage A CLL. The indications for treatment and the treatment regimens were based on current guidelines at the time of treatment, with most patients receiving an alkylating agent or purine analog. Biomarker studies and mutational analysis (*Online Supplementary Appendix*) were performed on samples stored at or within six months of diagnosis. Mutational analysis was performed at a second time point in 84 patients with a median of 72 months (range 24-145 months) between initial and subsequent testing (*Online Supplementary Table S2*). This study was implemented in accordance with the Declaration of Helsinki and has been ethically approved by the Regional Ethics Committee (REC).

At diagnosis, mutations were detected in *SF3B1* (16 of 199, 8%), *NOTCH1* (11 of 203, 5%), *POT1* (8 of 198, 4%), *XPO1* (2 of 172, 1%) *MYD88* (3 of 198, 1.5 %), *BIRC3* (1 of 197, 0.5%) and *FBXW7* (2 of 31 trisomy 12 cases, 6.5%). The majority of mutations have been previously observed in CLL, are annotated in COSMIC and predicted to have deleterious functional consequences based on PolyPhen and SIFT scores (*Online Supplementary Table S3*). These figures are broadly comparable to those in a recent large study of 1160 previously untreated patients of all stages of whom 82% were screened at diagnosis: *NOTCH1, SF3B1, XPO1, FBWX7* and *MYD88* mutations were found 12.3%, 9.0%, 3.4%, 2.5% and 1.5% of cases, respectively.[2] The clinical and biological features of patients with *NOTCH1*, *SF3B1* and low frequency mutations are shown in Figure 1A and *Online Supplementary Table S4*. The expected associations were apparent, such as both *NOTCH1* and *SF3B1* mutations being significantly associated with IGHV unmutated genes, high CD38 and ZAP70 expression.[4] Three of the 8 *POT1* mutated cases had mutated *IGHV* genes in contrast to previous data,[5] in which *POT1* mutations have occurred exclusively in cases with unmutated *IGHV* genes. In view of the reported increased incidence of telomere-containing

Table 1. Univariate Cox proportional hazard analysis of treatment-free and overall survival.

		Treatment-free survival/TFS (months)							Overall survival/OS (months)					
Mutation/biomarkers	status	total events		median TFS	95% CI	HR	95% CI	P	total events		median OS	95% CI	HR	95%CI	P
NOTCH1	wild type	137	52	105	88-128				190	91	117	107-133			
	mutated	8	8	58	26-126	3.9	1.8-8.2	<0.001	11	7	116	50-173	1.6	0.8-3.5	0.14
SF3B1	wild type	127	50	103	86-120				181	89	120	108-138			
	mutated	15	11	44	22-113	2.8	1.4-5.4	0.001	16	12	97	49-149	2.0	0.95-4.1	0.06
Low frequency mutations	wild type	106	34	113	91-127				154	74	122	112-143			
	mutated	7	6	42	11-67	8.6	3.3-22	<0.001	7	3	85	77-156	2.3	0.7-7.4	0.2
Gender	female	57	20	142	73-181				88	41	142	120-162			
	male	90	42	91	72-107	1.9	1.1-3.2	0.04	116	59	106	94-119	1.6	1.1-2.4	0.017
Disease	cMBL	65	20	107	74-124				89	40	118	107-143			
	CLL stage A	82	42	101	53-121	1.7	1.1-3.1	0.02	115	60	119	97-138	1.25	0.8-2.1	0.4
Disease progression	absent	85	1	121	102-152				137	56	119	103-138			
	present	62	61	44	30-101	144	20-1039	<0.001	67	44	116	102-150	1.7	1.1-2.5	0.009
IGHV-status	mutated	100	22	122	113-152				141	55	139	119-155			
	unmutated	47	40	34	22-53	10.7	6.1-19	<0.001	63	45	91	70-110	3.7	2.4-5.5	<0.001
CD38-status (30%)	negative	92	23	121	106-149				127	52	142	121-162			
	positive	43	30	68	42-84	4.8	2.7-8.5	<0.001	56	36	97	86-112	2.9	1.9-4.5	<0.001
ZAP70-Status	negative	83	21	122	109-152				119	53	141	120-162			
	positive	40	29	52	33-104	4.8	2.7-8.6	<0.001	50	31	107	85-121	2.4	1.5-3.8	<0.001
del(11q)	absent	128	44	112	89-123				175	79	121	112-140			
	present	12	11	19	4-86	7.2	3.6-14.3	<0.001	15	11	91	48-114	2.6	1.4-4.9	0.002
Trisomy 12	absent	89	37	118	103-143				124	64	131	116-162			
	present	26	16	81	49-132	1.9	1.1-3.5	0.026	40	24	108	78-142	1.8	1.1-2.9	0.02

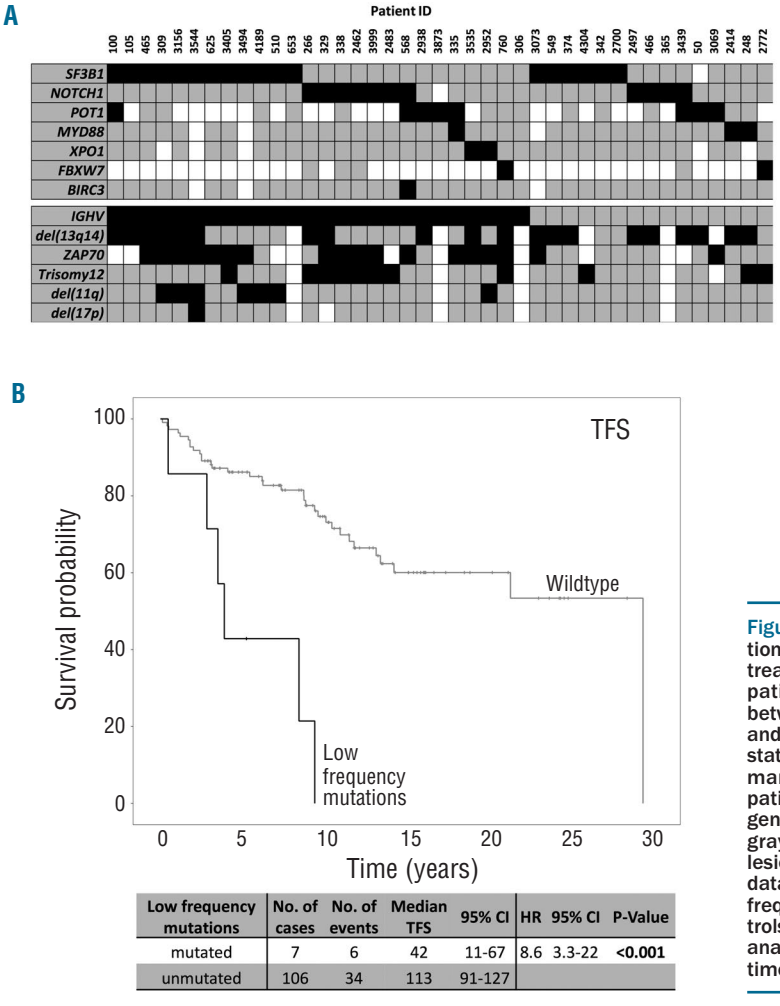
chromosome fusions in patients with *POT1* mutations, we examined the incidence of chromosomal complexity and instability in our 8 sequential cases with *POT1* mutations. Based on cytogenetic and FISH data at diagnosis and later time points, we detected a del(13q14) at the second time point only in 2 cases, but found no evidence of genomic complexity (*Online Supplementary Table S5*). Larger studies in patients pre- and post therapy, using more sensitive methods to detect complexity and instability, will be required to determine the biological significance of *POT1* mutations. The low incidence of the other mutations precluded any meaningful analysis of their associations with other biomarkers. We found no difference in the frequency of genomic mutations or any other biomarker, apart from a border-line higher incidence of CD38 expression in cMBL, between cases presenting with cMBL or stage A CLL. This is consistent with other recent data confirming the biological similarity between cMBL and Rai O CLL, including a similar incidence of *NOTCH1* and *SF3B1* mutations,[6] and provides justification for analyzing the outcome of the combined cMBL and stage A cohorts.

We then assessed the prognostic significance of mutations in our cohort. In view of the low frequency of all mutations other than *NOTCH1* and *SF3B1*, and the known favorable outcome of cases with an *MYD88* mutation,[7] we hypothesized that the collective analysis of the other low frequency mutations might provide insight into their biological importance and clinical utility in early stage disease.

Table 2. Multivariate Cox proportional hazard analysis of treatment-free and overall survival.

Variable	Treatment-free survival			Overall survival		
	HR	95% CI	*P*	HR	95% CI	*P*
Low frequency mutations	3.7	1.3-10.5	0.016	1.9	0.8-4.7	0.14
NOTCH1 mutations	1.4	0.5-4.0	0.5	1.1	0.5-2.7	0.8
SF3B1 mutations	1.9	0.9-4.3	0.1	1.3	1.02-5.3	0.045
Trisomy 12	1.5	0.7-3.2	0.3	1.2	0.7-2.1	0.6
del(11q)	3.7	1.5-8.6	0.003	1.3	0.6-2.7	0.5
IGHV-unmutated	5.7	2.7-11.9	<0.001	3.3	1.9-5.7	<0.001

TFS multivariate: 109 cases with 51 events, 97 cases with missing data; OS multivariate: 154 cases with 82 events, 52 cases with missing data.



Low frequency mutations	No. of cases	No. of events	Median TFS	95% CI	HR	95% CI	P-Value
mutated	7	6	42	11-67	8.6	3.3-22	**<0.001**
unmutated	106	34	113	91-127			

Figure 1. The associations between gene mutations, established biomarkers and time to first treatment in our series of cMBL and stage A CLL patients. (A) Shows the mutual relationship between gene mutations and other genetic lesions and biomarkers in CLL, sorted by IGHV mutational status. Rows correspond to specific lesions/biomarkers, and columns represent individual patients (only patients with mutations in the genes tested are shown). Boxes colored black and gray show the presence and absence of a lesion/biomarkers. A white box denotes that no data are available. (B) TFS for patients with 'low frequency' mutations compared to wild-type controls. The *P* value is derived from Kaplan-Meier analysis with a log rank test and median survival times with 95% confidence intervals.

Accordingly, we grouped mutations of *POT1, XPO1, BIRC3* and *FBXW7* together and compared the outcome of cases with any of these 'low frequency' mutations with those that were wild type for *NOTCH1*, *SF3B1* and the four 'low frequency' genes. In support of this hypothesis, we identified an enrichment of *NOTCH1* and *SF3B1* mutations in Stage A CLL and cMBL patients who ultimately developed progressive disease, and also showed that the collective presence of a low frequency mutation was significantly associated with subsequent disease progression [Odds Ratio (OR) 17.4, *P*=0.002] and need for treatment (OR: 18.0 and *P*=0.002) (*Online Supplementary Table S4*).

Univariate analysis confirmed recent data[8] showing that the presence of *NOTCH1* [(median 58 *vs.* 105 months; Hazards Ratio (HR) 3.9, *P*<0.001)], and *SF3B1* mutations (median 44 *vs.* 103 months; HR 2.8, *P*=0.001) were significantly associated with reduced treatment-free survival (TFS) but not overall survival (OS) (Table 1). However, the presence of a low frequency mutation was also associated with reduced TFS (median 42 *vs.* 113 months; HR 8.6, *P*<0.001) (Figure 1B).

We then estimated the impact of these 'low frequency' mutations on TFS and OS after controlling for confounding variables in multivariate Cox proportional hazard analysis depicted in Table 2. Along with the 'low frequency' mutations, other variables included in the analysis were *SF3B1* and *NOTCH1* status, trisomy 12, del(11q) and the presence or absence of mutated IGHV genes. Loss of chromosome 17p was omitted due to the very low frequency of del(17p) cases, all of which exhibited mutated *IGHV* genes.[9] As expected, unmutated IGHV genes remained the strongest predictor of poor treatment free (HR: 5.7, 95%CI: 2.7-11.9, *P*<0.001) and overall survival (HR: 3.3, 95%CI: 1.9-5.7, *P*<0.001). Loss of chromosome 11q was confirmed as an adverse prognostic factor in treatment-free survival (HR: 3.7, 95%CI: 1.5-8.6, *P*=0.003) and SF3B1 mutations showed border-line significance as an independent predictor of reduced overall survival (HR: 1.3, 95%CI: 1.02-5.3, *P*=0.045). In addition, our 'low frequency mutations' variable retained significance for reduced treatment-free survival (HR: 3.7, 95%CI: 1.3-10.5, *P*=0.016). While we recognize that the apparent poorer outcome of cases with low frequency mutations might reflect the presence of other undetected mutations or genomic instability rather than the mutations we detected, none had del(17p) or cytogenetic evidence of genomic complexity and only one patient showed a del(11q).

Finally, 84 patients were screened sequentially for mutations compared to those screened only at diagnosis in order to document clonal evolution in CLL. The sequential and single time point cases are shown in *Online Supplementary Table S2* and no significant differences were observed between the two groups, apart from a border-line higher frequency of trisomy 12 in the cases tested only at diagnosis. Among the sequential cases, 47 patients (56%) remained stable during observation time, but 37 (44%) progressed and 36 patients received treatment between diagnosis and second testing. We detected mutations of *SF3B1* in 4 cases and XPO1 in one case, not found on screening at diagnosis. Of these, 3 presented with cMBL, and 2 with Stage A CLL and 3 out of 5 had progressive disease. Additional characteristics of these patients are shown in *Online Supplementary Table S6*. Although the number of patients screened was small, these results were consistent with those of a recent large multinational study in which the incidence of *SF3B1*, but not *NOTCH1*, mutations rose with increasing time from diagnosis to the date of sampling.[8]

In summary, our study suggests that screening for these low frequency mutations may have utility in the clinical management of early stage CLL and cMBL, and future larger studies should evaluate the incidence and clinical significance of low frequency potential driver mutations in early disease to assess their relevance in new molecular prognostication systems.

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