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White blood cell count at diagnosis and immunoglobulin variable region gene (IGHV) mutations are independent predictors of treatment-free survival in young patients with stage A chronic lymphocytic leukemia

by Ilaria Del Giudice, Francesca Romana Mauro, Maria Stefania De Propris, Simona Santangelo, Marilisa Marinelli, Nadia Peragine, Valeria Di Maio, Mauro Nanni, Rita Barzotti, Francesca Mancini, Daniele Armiento, Francesca Paoloni, Anna Guarini, and Robin Foa'

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White blood cell count at diagnosis and immunoglobulin variable region gene (IGHV) mutations are independent predictors of treatment-free survival in young patients with stage A chronic lymphocytic leukemia

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Abstract

A comprehensive panel of clinical-biologic parameters was prospectively evaluated at presentation in 112 patients with chronic lymphocytic leukemia (<65 years), to predict the risk of progression in early stage disease. Eighty-one% were in Binet stage A, 19% in stages B/C. Treatment-free survival was evaluated as the time from diagnosis to first treatment, death or last-follow-up. In univariate analysis, advanced stage, hemoglobin, platelets, white blood cell, leukemic lymphocyte count, raised beta 2-microglobulin and LDH, unmutated immunoglobulin variable region genes, CD38, del(17p), del(11q) and +12, were significantly associated with a short treatment-free survival; the T/leukemic lymphocyte ratio was associated with a better outcome. Multivariate analysis of treatment-free survival in stage A patients selected a high white blood cell count and unmutated immunoglobulin variable region genes as unfavorable prognostic factors and a high T/leukemic lymphocyte ratio as a favorable one. At diagnosis, these parameters independently predict the risk of progression in stage A chronic lymphocytic leukemia patients.

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INTRODUCTION

Although Binet and Rai clinical staging systems for chronic lymphocytic leukemia (CLL) remain the most powerful prognosticators to identify advanced stages for which treatment-free survival (TFS) and overall survival (OS) are usually short, they provide no risk stratification in early stages, nowadays the most represented at diagnosis. Binet stage A CLL patients normally undergo clinical observation up to disease progression and treatment requirement, for a time frame ranging from a few months up to decades.[1-3]

A number of new phenotypic, molecular and genetic parameters in addition to the traditional clinical features have enabled clinicians to better predict TFS and OS of CLL patients.[1,4,5] However, it is still not clear which is the relative importance of the various clinical-biologic parameters in the assessment of early stage CLL prognosis.[5]

In 2002, two multivariate analyses[6,7] showed that the immunoglobulin heavy chain variable region gene (IGHV) mutational status, *TP53* abnormalities and in one study also del(11q)[6] were independent predictors of OS in stage A CLL. On the contrary, CD38 had no significant impact. In 2003, ZAP-70 expression was proposed as a surrogate marker of unmutated IGHV,[8] although 20-30% of “discordant” cases were recognized. Since then, a number of studies have tried to demonstrate the superiority of a given parameter in terms of prognostication.[8-12]

The timing of the evaluation of these parameters is also important, as it is now clear that genetic abnormalities can be acquired over time.[13]

In this study, we assessed the distribution and clinical significance of a comprehensive panel of clinical-biologic parameters prospectively evaluated at diagnosis in all young patients sequentially diagnosed with CLL at our Institution, focusing on their predictive impact on the progression of early stage CLL.

DESIGN and METHODS

Patients

From November 2002 to December 2008, 112 young patients (<65 years) diagnosed with CLL at our Institution were included in the study. There were 62 males and 50 females, with a median age of 52 years (range 29 - 68). Eighty-one% were in Binet stage A, 19% were in stages B/C. Rai stage 0 was recorded in 61% of patients, I/II in 33.5%, III/IV in 5.5%.

Clinical features included: age, gender, Binet and Rai stage, hemoglobin (Hb), platelets (PLTs), WBC, lymphocyte counts, amount and distribution of T cells, amount of CLL cells. Serological parameters included: beta2-microglobulin (β2-m), LDH, IgG, IgA and IgM levels. Biologic parameters included: lymphocyte morphology, IGHV mutations, CD38, ZAP-70 expression, cytogenetic abnormalities evaluated by fluorescence in situ hybridization (FISH) and *TP53* gene sequencing.

Details on the immunophenotype, IGHV mutations,[14,15] *TP53* sequencing[16] and FISH analyses[15] are available in the *Online Supplementary Appendix.*

Statistics

Prognosis was evaluated as TFS, calculated from the time of diagnosis to the first treatment, death or last follow-up. Since no patient died before treatment, the TFS probability has been estimated using the Kaplan-Meier method, instead of the Cumulative Incidence Estimation, considering death before treatment as competing risk- and using the Log-rank test to evaluate differences between factors.

The Cox model has been used for the multivariate analysis; first, all factors with a clinical relevance or a statistical significance/trend for significance ($p \leq 0.07$) were included in the model; subsequently, factors which lost significance and considered less important by a clinical prospective were excluded by the model. The WBC count and the T/CLL ratio cut-off points were estimated by means of martingale residuals.

RESULTS and DISCUSSION

Clinical and biologic features of CLL at diagnosis

The distribution of the clinical-biologic markers of all 112 patients and of 90 stage A CLL is summarized in Table 1 (and in supplementary Table 1). We focused our study on patients younger than 65 years because of the importance of prognostic information in individuals with a long life expectancy, whose expected survival is more affected by the direct effect of the disease than in older patients.[17] However, our results could be applied to the entire CLL population, as no difference related to age is reported regarding the presence of unmutated IGHV or del(17p)[6], the prognostic value of ZAP70, IGHV, CD38,[9] and the CD49d expression.[18] Moreover, age appears to affect more OS than TFS.[6,12,18-21]

Unmutated IGHV cases represented 36% of the overall cohort and 25% of stage A CLL. CD38+ ($\geq$7%) cases were 26% and 20%, and ZAP-70+ ($\geq$20%) were 32% and 27% in the two cohorts, respectively. Thus, unfavorable biologic features such as unmutated IGHV, CD38 and ZAP-70 positive expression, can be found in about one third of young CLL at diagnosis and not exclusively in the advanced stages.

Contrariwise, prognostically adverse FISH abnormalities are present only in a small subgroup of cases at diagnosis. The incidence of del(17p) (cut off >20% cells) and del(11q) (cut off >10% cells) was 2% and 8% in the entire cohort and 0% and 5% in stage A CLL, respectively. The 11 patients with del(17p) or del(11q) all showed unmutated IGHV, ZAP-70+ in 8/11 and CD38+ in 7/11. In the analysis, they were pooled with the 8 trisomy 12 cases, for their numerical dearth.

TP53 mutation was present in 4 cases (3.6%), 2 in stage A: 3 showed unmutated IGHV and del(17p), and 1 mutated IGHV and no del(17p).

The median WBC count at presentation was not different between the entire cohort and stage A CLL, being 18.4 and 17 x 10^9/l, respectively. The median number of total lymphocytes, CLL cells, and T cells was 11.7 and 11.1 x 10^9/l, 8.8 and 8.6 x 10^9/l, and 1.9 and 1.9 x 10^9/l, respectively, in the two groups.

It is notable that raised β2-m and LDH levels were present only in 5% and 17% of the entire cohort, and in 1% and 7% of stage A CLL, questioning the real utility of β2-m in identifying early stages CLL at high risk of progression. Hypogammaglobulinemia was present in a notable proportion of patients (Table 1).

Risk factors for TFS in univariate analysis

The relevance of clinical and biologic markers as predictors of TFS, was investigated in univariate and multivariate analysis. During the follow-up (median 35.4 months, range 1.1-93.8), 46 patients (41%) underwent treatment. The median TFS was 45.2 months (supplementary Figure 1) in the entire cohort and 62.4 months in stage A CLL.

In univariate analysis (Table 2A), the following variables resulted associated with a short TFS: advanced stage Binet B/C and Rai intermediate/high (<0.0001), Hb, PLTs, WBC count (<0.0001) as continuous variables, raised β2-m (<0.0001) and LDH (<0.0001), unmutated IGHV (<0.0001), CD38+ (<0.0001), adverse cytogenetic abnormalities (del(17p)>20%, del(11q)>10%, +12) (<0.0001). The absolute CLL lymphocyte count was also a significant adverse prognostic parameter (<0.0001), reflecting the impact of disease burden, whilst the T/CLL lymphocyte ratio was a significant favorable variable (0.0004). The T-lymphocyte count showed a strong positive correlation with the WBC (Pearson's correlation coefficient=0.68347, p<.0001) and was not included in the analysis.

Atypical CLL morphology and ZAP-70+ ($\geq$ 20%) were not significant (p 0.07 for both), as well as gender, age, hypo IgG and the CD4/CD8 ratio. The CD4/CD8 ratio was inverted only in 3 cases.

Particularly, TFS was significantly shorter in cases with unmutated versus mutated IGHV (at 36 months, 24.8% and 77%, respectively p<.0001) (Figure 1A) and CD38+ versus CD38- (at 36 months 32.5% vs. 67.9%, p<.0001) (supplementary Figure 2).

Cases with del(17p) (>20% cells) or del(11q) (>10% cells) had a TFS at 36 months of 0% and 15.6% versus 41.7% for cases with +12, 66% for cases with no abnormalities and 66.4% for cases with del(13q) (p<.0001) (supplementary Figure 3). FISH abnormalities evaluated with a lower laboratory cut-off were much less significant (p 0.0121), as patients with del(17p)>5% had a TFS at 36 months of 77.5% (median 47.1 months). Thus, we prefer to consider the higher cut-off for prognostic purposes related to TFS.

We also explored different cut-off point for ZAP-70 and CD38 expression. ZAP-70 $\geq$10%, present in 38.3% of cases, was more significant for TFS (p 0.0021) than ZAP-70 $\geq$20%. The CD38 $\geq$30% was as significant as $\geq$7% for TFS, but present only in 19% of all cases and in 13% of stage A, as previously described.[22]

The results of this univariate analysis are mostly in agreement with others published, with the exception of ZAP-70.

Multivariate analysis of TFS in stage A CLL

The multivariate analysis of TFS was focused on the 90 patients with Binet stage A, those for whom the prediction of progression is most relevant, and included age, WBC count, Hb, PLTS, T/CLL lymphocytes ratio (as continuous variables), LDH, morphology, ZAP-70 and CD38 expression, IGHV mutations and FISH (del(13q) vs. normal vs. tris12+del(17p)>20%+del(11q)>10%).

High WBC count and unmutated IGHV resulted as independent unfavorable prognostic factors (Table 2B, model 1). From this model, we excluded the WBC count to verify whether this parameter could show a masking effect on the T/CLL lymphocytes ratio, due to the strong correlation between these variables. Excluding the WBC, a high T/CLL ratio was significantly associated with a better outcome (Table 2B, model 2); the IGHV status was still significant.

We also performed a multivariate analysis excluding the IGHV status to identify a "simplified" prognostic model. WBC count and LDH emerged as the independent prognostic parameters of TFS (Table 2B, model 3) in stage A CLL; this model could be easily employed in developing countries or wherever molecular biology facilities are not available.

Thus, we searched for a significant cut-off point for WBC and T/CLL ratio. A WBC count greater than 30 $x10^9$/l and T/CLL ratio less than 0.2 significantly identified patients with a short TFS (Figure 1B-C).

Excluding the WBC and T/CLL lymphocytes ratio and considering the absolute CLL cells number, the latter was an unfavorable significant factor in both the entire cohort (HR: 1.014, 95%CI: 1.002-1.026; p=0.0246) and in stage A cases (HR: 1.069, 95%CI: 1.039-1.099; p=<.0001 - data not shown).

The WBC count is always an independent prognostic marker in multivariate analysis including pure clinical[19-21] or clinical-biologic parameters of CLL.[6] However, this simple measure of tumor burden is often not considered, at variance from the prognostic studies in acute leukemias, where it is invariably included. Our WBC cut-off of 30 $x10^9$/l is a more than reasonable proposal when compared to the 30 $x10^9$/l lymphocyte count limit which characterizes the "smoldering CLL"[23] or the A' and A'' subgroups,[24] as well as the other proposed risk categories to stratify CLL patients.[19-21] Our results on the T-cell compartment are strengthened by recent similar observations,[25] underlining the importance of the non-malignant host immune compartment in the evolution of the

disease. In our study, neither ZAP-70, CD38 (with both cut-off values) nor FISH showed an independent prognostic value, whilst the significant impact of IGHV was reinforced.[6,7] The scarce number of stage A CLL with del(17p) or del(11q) might account for the lack of significance of cytogenetics in our series. Whilst CD38 does not retain an independent prognostic value when IGHV mutations or other markers are considered,[6,7,9,12,18] the value of ZAP-70 is proven by some studies,[9] but not by ours and by others.[18]

Our findings present some limitations. First, a longer follow-up is necessary to further validate our prognostic model as predictor of progression and survival for stage A CLL. Second, our sample size is relatively small and our results represent a proposal that needs to be confirmed in independent larger series. The topic is of interest, as witnessed by the recent efforts of the MDACC[19] to define a widely applicable CLL prognostic index based on the clinical variables of age, gender, β2-m, lymphocyte count, stage and number of involved lymph nodal groups (independently validated by the Mayo Clinic[20] and the Italian GIMEMA group[21]) and by ongoing European prospective studies on stage A CLL prognostication based on clinical and biologic markers.

In conclusion, in a young patient with stage A CLL diagnosis, the IGHV mutational status, WBC count and T/CLL lymphocyte ratio are the most important parameters to predict TFS. Our results meet the need of dissecting CLL Binet stage A patients into different prognostic groups and may question the utility of performing FISH analysis in the work-up of these patients at first diagnosis, rather than at progression before treatment, as stated in the new IWCLL guidelines.[26]

AUTHORSHIP and DISCLOSURES

IDG designed research, collected and analyzed the data and wrote the manuscript; FRM analyzed patients and critically contributed to the revision of the manuscript; MSDP performed immunophenotype; SS performed molecular analysis of IGHV genes rearrangements; MM performed molecular analysis of *TP53* gene mutations; NP and VDM collected and centralized patients samples; MN and RB performed FISH analysis; FM performed peripheral blood morphology; DA analyzed patients; FP performed the statistical analysis; AG analyzed the data and contributed to the revision of the manuscript; RF reviewed the design of the study, analyzed and discussed the results, and critically revised the manuscript.
The authors reported no potential conflict of interest.

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Table 1. Biologic and clinical characteristics of 112 CLL at diagnosis.

Characteristics at diagnosis	All cases (N=112)	Stage A (N=90)
Biological variables		
IGHV mutated (≤98%) *n/N (%)*	70/109 (64.2)	67/89 (75.3)
IGHV unmutated (>98%) ***n/N (%)***	**39/109 (35.8)**	**22/89 (24.7)**
Del(17p) >20% ***n/N (%)***	**2/111** (1.8)	0 (0)
Del(11q) >10% *n/N (%)*	9/111 (8.1)	5/90 (5.6)
+12 >5% *n/N (%)*	8/111 (7.2)	5/**90 (5.6)**
Del(13q) >5% isolated ***n/N (%)***	**61/111 (55.0)**	**55/90 (61.1)**
Normal FISH (none of the above) *n/N (%)*	31/111 (27.9)	25/90 (27.8)
***TP53* mutation** *n/N (%)*	4/111 (3.6)	2/90 (2.2)
***TP53* inactivation by mutation and/or deletion** n/N (%)	6/111 (5.4)	2/90 (2.2)
CD38 >7% *n/N (%)*	29/111 (26.1)	18/90 (20.0)
CD38 >30% *n/N (%)*	21/111 (19)	12/90 (13.3)
ZAP-70 ≥20% *n/N (%)*	34/107 (31.8)	23/86 (26.7)
ZAP-70 ≥10% *n/N (%)*	41/107 (38.3)	25/86 (29.1)
Atypical morphology *n/N (%)*	27/111 (24.3)	17/90 (18.9)
Clinical variables		

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Characteristics at diagnosis	All cases (N=112)	Stage A (N=90)
Age *median (range)*	52 (29-68)	53 (29-62)
Hb (g/dl) *median (range)*	14 (7.5-16.9)	14.2 (11.4-16.9)
PLTs (x10^9/l) *median (range)*	213 (47-406)	219 (111-406)
WBC (x10^9/l) *median (range)*	18.4 (5.8-236.6)	16.9 (5.79-101.1)
Lymphocytes (x10^9/l) *median (range)*	11.7 (2.7-232)	11.1 (2.74-94)
CLL lymphocytes (x10^9/l) *median (range)*	8.8 (1.7-224.9)	8.6 (1.7-86.5)
CD3+ cells (x10^9/l) *median (range)*	1.9 (0.37-16.6)	1.9 (0.38-3.76)
CD4/CD8 (<1) *median (range)*	3/107 (2.8)	3/88 (3.4)
T/CLL lymphocytes ratio *median (range)*	0.18 (0.01-1.09)	0.19 (0.01-1.09)
Gender: Male *n/N (%)*	62/112 (55.4)	50 (55.6)
Rai stage *n/N (%)*		
0	68/111 (61.3)	68 (75.6)
I	22/111 (19.8)	17 (18.9)
II	15/111 (13.5)	5 (5.5)
III	2/111 (1.8)	-
IV	4/111 (3.6)	-
Binet stage *n/N (%)*		
A	90/111 (81.1)	90 (100)
B	15/111 (13.5)	-
C	6/111 (5.4)	-
Raised β2-m (>3400 ng/l) *n/N (%)*	5/109 (4.6)	1/88 (1.1)

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Characteristics at diagnosis	All cases (N=112)	Stage A (N=90)
Raised LDH (>190 U/l) *n/N (%)*	19/111 (17.1)	6/89 (6.7)
Hypo IgG (<700 mg/dl) *n/N (%)*	22/105 (21.0)	14/86 (16.3)
Hypo IgA (<70 mg/dl) *n/N (%)*	18/105 (17.1)	12/86 (14.0)
Hypo IgM (<40 mg/dl) *n/N (%)*	42/105 (40.0)	31/86 (36.0)

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Table 2A. Biologic and clinical risk factors for TFS identified by univariate analysis at CLL diagnosis.

		TFS at 36 months (C.I. 95%)	p-value
Gender	**Female**	61.9% (52.8-72.6)	0.8002
	Male	56.7% (49.1-65.4)	
Binet stage	**A**	73.3% (65.6-81.8)	<0.0001
	B	0.0%	
	C	0.0%	
Rai stage	**Low**	75.0% (66.3-84.9)	<0.0001
	Intermediate	36.7% (30.6-44.1)	
	High	0.0%	
β2-m *(at 12 months)*	**Normal**	89.8% (84.5-95.4)	<0.0001
	Raised	20.0% (14.1-28.4)	
LDH *(at 24 months)*	**Normal**	78.6% (71.5-86.3)	<0.0001
	Raised	21.6% (17.0-27.3)	
Hypo-IgG	**No**	67.3% (59.6-76.0)	0.1461
	Yes	40.9% (32.6-51.4)	
CD38/CD19 (>7%)	**Negative**	67.9% (60.3-76.4)	<0.0001
	Positive	32.5% (26.0-40.7)	
CD38/CD19 (>30%)	**Negative**	66.6% (59.5-74.6)	<0.0001
	Positive	25.3% (19.5-32.7)	
CD3	**<5%**	29.0% (23.0-36.5)	0.0002
	6-15%	52.0% (44.4-60.8)	
	16-30%	84.5% (74.4-95.9)	
	>30%	91.7% (78.4-100.0)	
ZAP-70 (≥10%)	**Negative**	69.4% (60.6-79.5)	0.0021
	Positive	41.1% (34.7-48.7)	
ZAP-70 (≥20%)	**Negative**	64.4% (56.4-73.5)	0.0764
	Positive	45.4% (37.6-54.8)	
Morphology	**Typical**	62.7% (55.4-71.0)	0.0698
	Atypical	49.3% (40.0-60.6)	
Cytogenetic aberration	**del17p>20%**	0.0%	<0.0001
	del11>10%	15.6% (11.8-20.5)	

	Trisomy 12	41.7% (27.9-62.3)	
	del13q	66.4% (57.6-76.5)	
	Normal	66% (54.1-80.4)	
IGHV	**Mutated**	77% (68.6-86.4)	<0.0001
	Unmutated	24.8% (21-29.3)	
		Hazard Ratio (95% CI)	**p -value**
Age		1.026 (0.984-1.07)	0.2314
Hb g/dl		0.747 (0.62-0.901)	0.0023
PLTS x10^9/l		0.993 (0.988-0.997)	0.0033
WBC x10^9/l		1.014 (1.01-1.019)	<0.0001
CLL lymphocytes x10^9/l		1.002 (1.002-1.003)	<0.0001
CD3+ lymph/CLL lymph ratio		0.007 (0.000-0.109)	0.0004
CD4/CD8		1.074 (0.787-1.466)	0.6538

Table 2B. Multivariate Cox regression analysis of TFS in stage A CLL.

Model 1: results from a multivariate analysis including WBC count, T/CLL lymphocyte ratio (as continuous variables), LDH, IGHV mutation status.

Model 2: multivariate analysis excluding the WBC count.

Model 3: multivariate analysis excluding the IGHV mutational status ("simplified" prognostic model).

Model 1		
Variable	**Hazard Ratio (95% CI)**	**p-value**
WBC x10^9/l	1.052 (1.022-1.082)	0.0005
LDH: pos vs. neg	3.212 (0.651-15.851)	0.1519
T/CLL ratio	0.105 (0.002-5.079)	0.2544
IGHV: unmutated vs. mutated	2.713 (1.146-6.42)	0.0232
Model 2		
Variable	**Hazard Ratio (95% CI)**	**p-value**
T/CLL ratio	0.001 (0.000-0.047)	.001
LDH: pos vs. neg	2.82 (0.569-13.99)	0.2045
IGHV: unmutated vs. mutated	3.057 (1.302-7.178)	0.0103
Model 3		
Variable	**Hazard Ratio (95% CI)**	**p-value**
WBC x10^9/l	1.053 (1.025-1.082)	0.0002
LDH: pos vs. neg	5.147 (1.108-23.906)	0.0365
T/CLL ratio	0.095 (0.002-4.949)	0.2430

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Figure 1A. TFS in CLL patients according to the IGHV mutational status. TFS of 39 IGHV unmutated and 70 IGHV mutated CLL patients.

Figure 1B. TFS in CLL patients according to the WBC count. TFS of 33 CLL patients with a WBC $\geq 30x10^9$/l and 79 with a WBC $<30x10^9$/l.

Figure 1C. TFS in CLL patients according to T/CLL ratio. TFS of 62 CLL patients with a T/CLL ratio <0.2 and 50 with a T/CLL ratio $\geq$0.2.

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Figure 1A.

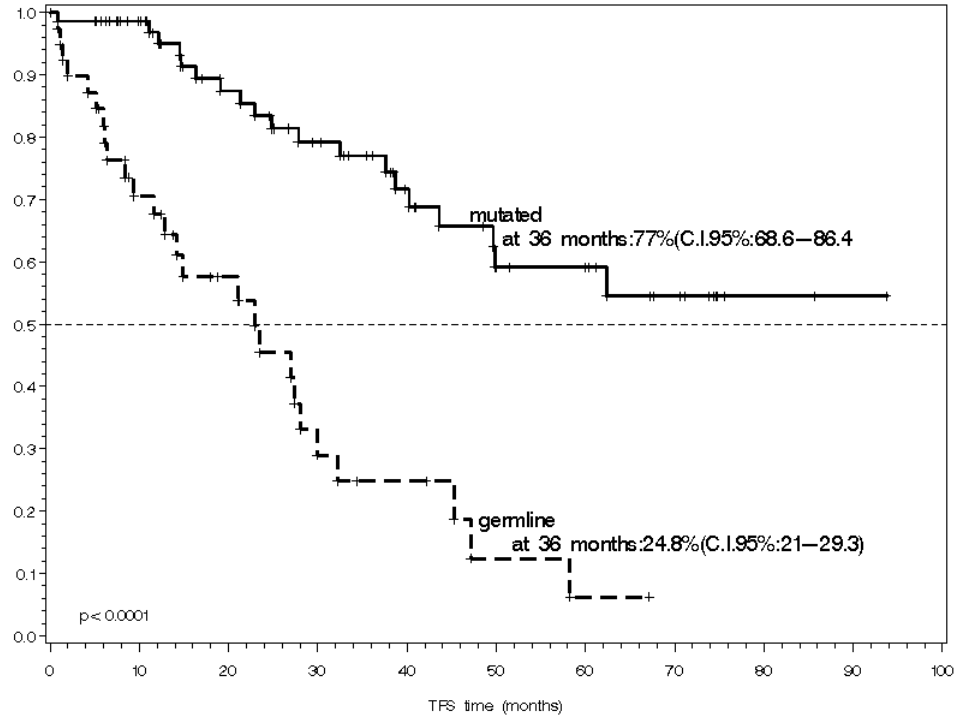

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Figure 1B.

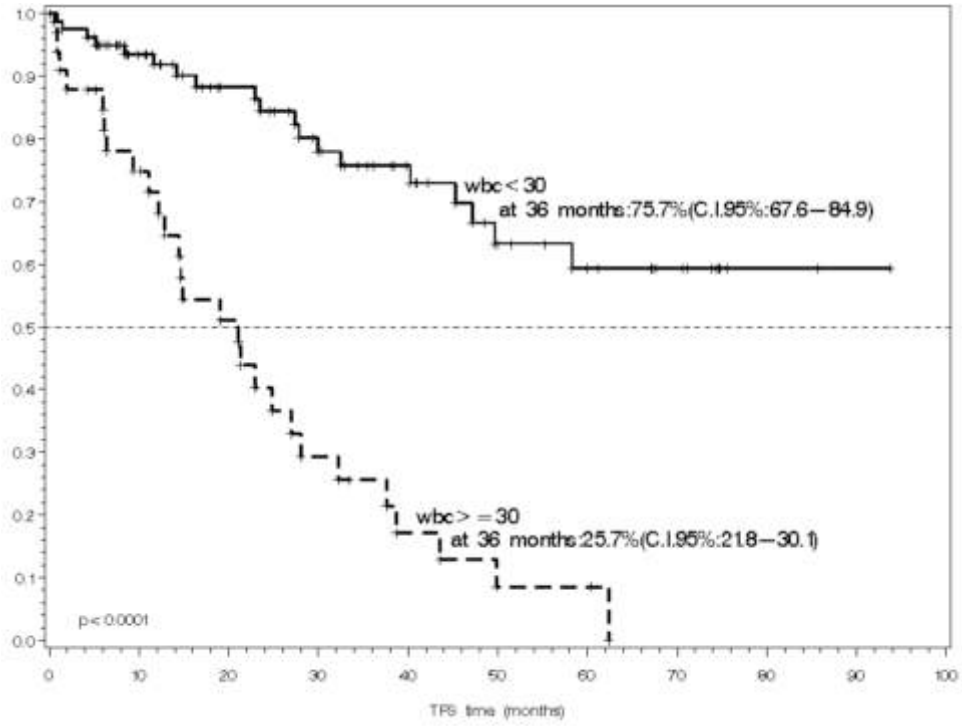

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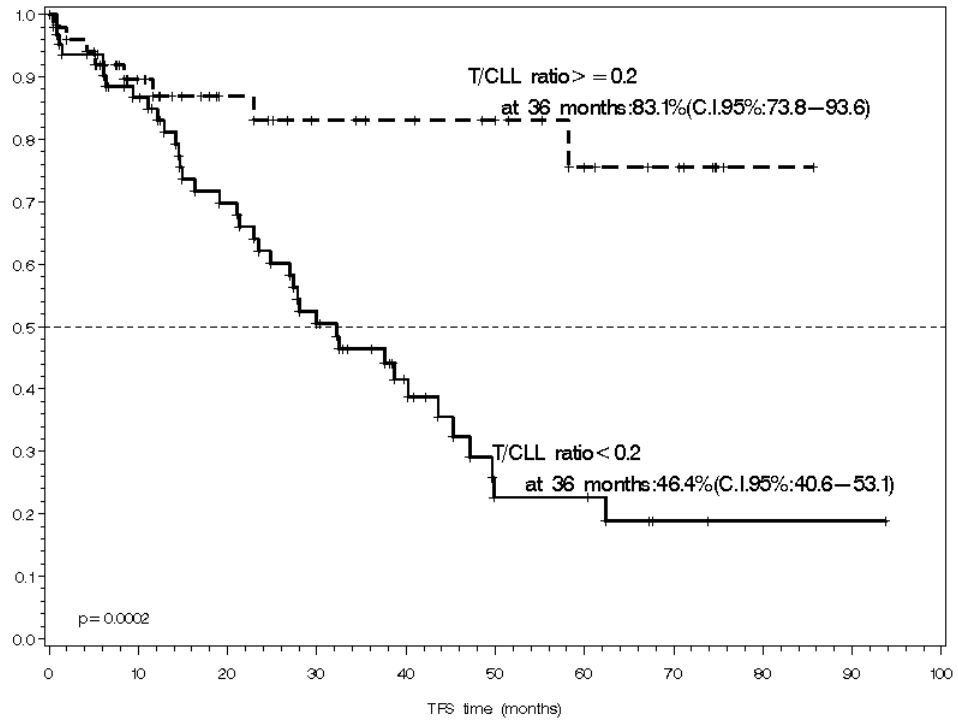
Figure 1C.



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Online Supplementary Appendix

Immunophenotype

Fresh peripheral blood cells were characterized using four-color immunostaining by flow cytometry with a FACSCalibur flow cytometer (Becton Dickinson, Immunocytometry Systems, San Josè, CA, USA) and the Cell Quest software (Becton Dickinson, Immunocytometry Systems). The panel included the following monoclonal antibodies (mAbs): CD5-phycoerythrin (PE), CD20-fluorescin isothiocyanate (FITC), CD22-PE, CD2-FITC, CD3-FITC, CD4-allophycocyanin (APC), CD8-PE, CD79b-FITC, CD56-FITC, CD16-PE (from Becton Dickinson, Immunocytometry Systems); CD18-FITC, CD11a-FITC (Caltag Laboratories, San Francisco, CA, USA); FMC7-FITC, anti-kappa chain-PE, anti-lambda chain-FITC, CD23-FITC (from Dako, Glostrup, Danmark); anti-CD19-phycoerythin/cyanin 5·1 (PC5) (from Beckman/Coulter, Miami, FL, USA).

The expression of CD38 was evaluated utilizing a quadruple staining combination with CD2-FITC/CD38-PE/CD19-PerCP/CD5-APC antibodies (from BD Biosciences, San Jose, CA). For ZAP-70 detection, the cells were fixed and permeabilized with the Fix and Perm product (Caltag) and stained utilizing the quadruple combo: ZAP-70-Alexa488Fluor (Caltag)/CD7-PE/CD19-PerCP/CD5-APC (BD Biosciences). Antigen expression was considered positive if $\geq$20% of the leukemic cells expressed the ZAP-70 molecule and $\geq$7% CD38.

PCR amplification of immunoglobulin rearrangements and sequence analysis

Genomic DNA was extracted from leukemic cells using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI, USA), according to the manufacturer's instructions. IgHV-D-J gene rearrangements were amplified by polymerase chain reaction (PCR) from genomic DNA using Taq polymerase with family-specific primes hybridizing to leader or framework (FR) 1 sequences and J_H, as previously described.[14,15] PCR products were size-selected by electrophoresis in 2% agarose gel containing 10 mg/ml ethidium bromide (SIGMA-ALDRICH, St. Louis, MO). The expected products were excised from the agarose gel and purified by MinElute Gel Extraction Kit (QIAGEN, Valencia, CA, USA).

Sequencing was performed using an automatic ABI PRISM 3100 AVANT DNA genetic analyzer (Applied Biosystem, Foster City, CA,USA). IgHV-D-J nucleotide sequences were aligned to the V-BASE sequence directory using the Mac Vector software version 6.0.1 (Oxford Molecular Group, Oxford, United Kingdom), to the IgBLast data base (*http//www.ncbi.nlm.nih.gov/igblast/,* National Cancer for Biotechnology Information, Bethesda, MD, USA) and to the international ImMunoGeneTics information system (*http://imgt.cines.fr*, Initiator and Coordinator: Marie-Paule Lefranc, Montpelier, France). Sequences were considered unmutated if deviation from the closest germline gene was <2%.

DNA sequencing analysis of TP53

TP53 exons 5, 6, 7 and 8 were amplified by PCR from genomic DNA using AmpliTaq Gold polymerase (Applied Biosystems), as previously described.[20] PCR amplification products were assessed by electrophoresis in 2% agarose gels containing 10 mg/ml ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). The expected products were excised from the agarose gel and purified using the MinElute Gel Extraction Kit (Qiagen). Sequencing was performed using the automatic ABI PRISM 3100 AVANT DNA genetic analyzer (Applied Biosystems). Sequence data were compared with wild-type sequences and the identified mutations were confirmed by duplicate PCR and sequence analysis.

FISH

FISH analysis was performed as previously described.[21] The 13q14 region was evaluated with the D13S25 probe (Vysis, Downers Grove, IL), the 11q23 region with the *ATM* probe (Vysis), chromosome 12 with an α -satellite probe (Oncor, Gaithersburg, MD) and the 17p13 region with a cosmidic probe (Oncor). A laboratory cut-off of >5% for all the probes and a clinical cut-off of >20% for del(17p) and >10% for del(11q) were utilized to define the presence of a genetic alteration.

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Supplementary Table 1. FISH abnormalities with different cut-off levels in 112 CLL at diagnosis.

Characteristics at CLL diagnosis	All cases (N=112)	Stage A (N=90)
Del(17p) >5%* *n/N (%)*	15/111 (13.5)	13 (14.4)
Del(11q) >5% *n/N (%)*	13/111 (11.7)	7 (7.8)
+12 >5% *n/N (%)*	7/111 (6.3)	5 (5.6)
Del(13q) >5% isolated *n/N (%)*	49/111 (44.1)	43 (47.8)
Normal FISH (none of the above) *n/N (%)*	27/111 (24.5)	22 (24.4)

*del(17p) 6% in 8 cases; del(17p) 7% in 2 cases; del(17p) 8% in 1 case: del(17p) 11% in 1 case; del(17p) 14% in 1 case; del(17p) 50% in 1 case, del(17p) 62% in 1 case.

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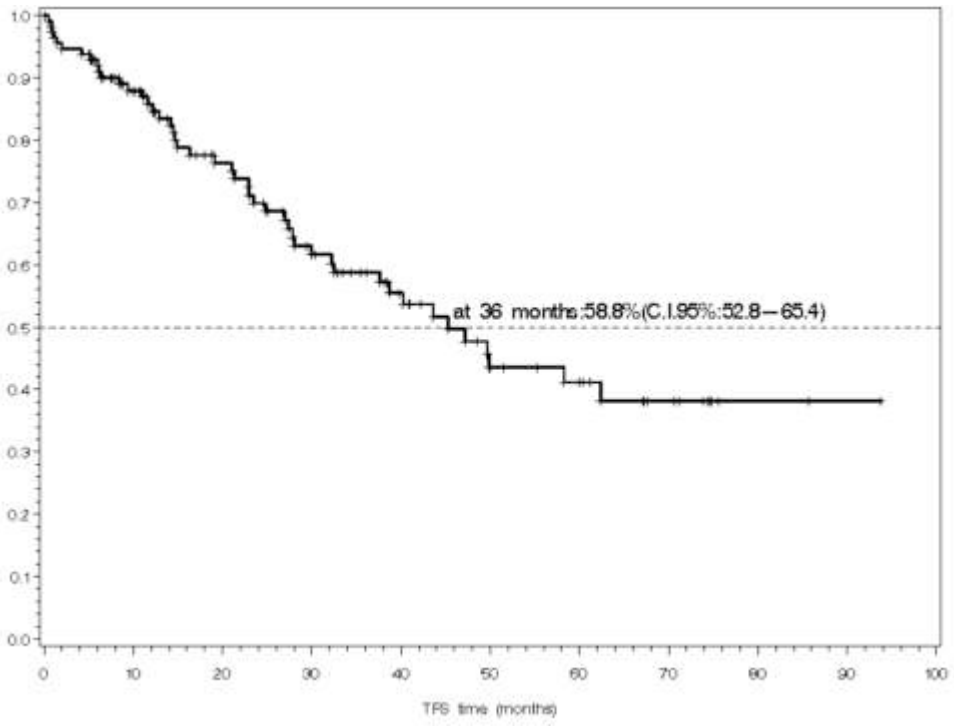
Supplementary Figure 1. TFS in CLL patients from diagnosis. TFS of 112 young CLL patients (median 45.2 months).



Supplementary Figure 2. TFS in CLL patients according to the CD38 expression. TFS of 29 CD38+ (>7%) and 82 CD38- CLL patients.

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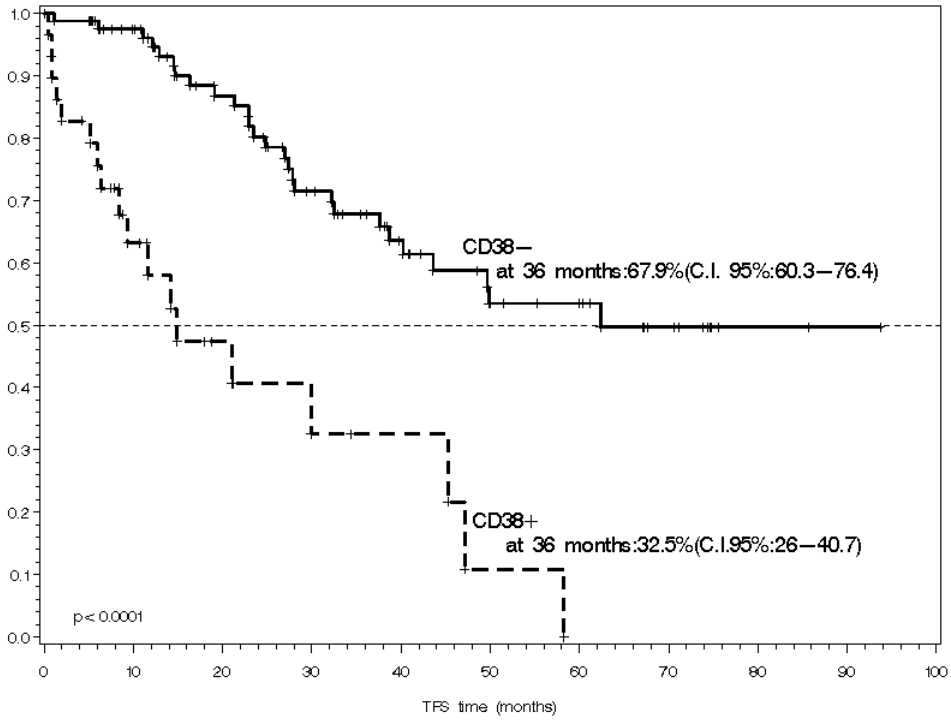

Supplementary Figure 3. TFS in CLL patients according to FISH genetic abnormalities. TFS of 19 patients with del(17p) (2 cases), del(11q) (9 cases), +12 (8 cases) and 92 patients with del(13q) or no abnormalities.

