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

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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) 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); 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, FLO).

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 ≥20% of the leukemic cells expressed the ZAP-70 molecule and ≥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), 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 JH, 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).

Sequencing was performed using an automatic ABI PRISM 3100 AVANT DNA genetic analyzer (Applied Biosystem, Foster City, CA). 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) 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). 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.

Online Supplementary Table S1. FISH abnormalities with different cut-off levels in 112 CLL at diagnosis.

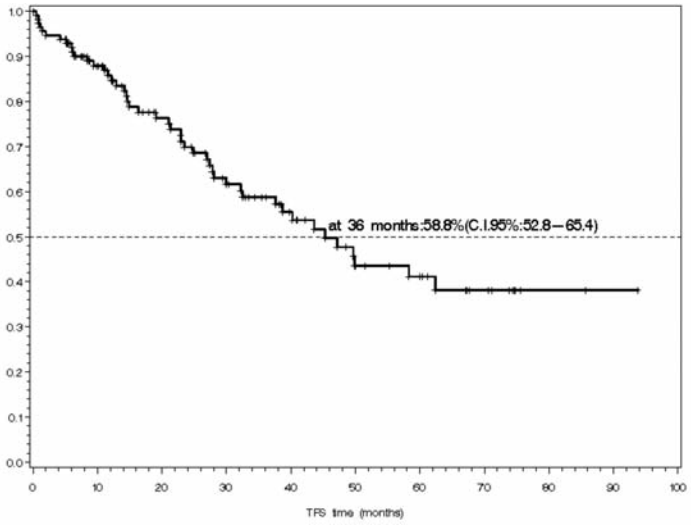
Characteristics at CLL diagnosis	All cases (N=112)	Stage A (N=90)
Del(17p) >5%* n/N (%)	15/111 (13.5)	13 (14.4)
Del(11q) >5% n/N (%)	13/111 (11.7)	7 (7.8)
+12 >5% n/N (%)	7/111 (6.3)	5 (5.6)
Del(13q) >5% isolated n/N (%)	49/111 (44.1)	43 (47.8)
Normal FISH (none of the above) n/N (%)	27/111 (24.5)	22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

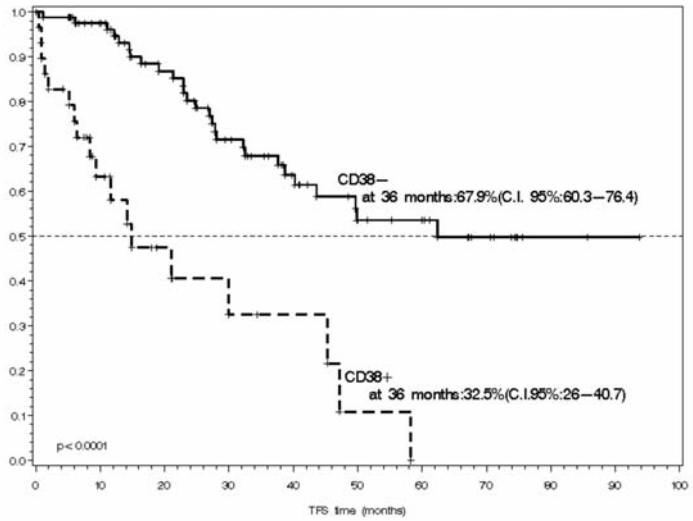
Online Supplementary Table S2. Biologic and clinical risk factors for TFS identified by univariate analysis at CLL diagnosis.

		TFS at 36 months (C.I. 95%)	*P* value
Gender	Female Male	61.9% (52.8-72.6) 56.7% (49.1-65.4)	0.8002
Binet stage	A B C	73.3% (65.6-81.8) 0.0% 0.0%	<0.0001
Rai stage	Low Intermediate High	75.0% (66.3-84.9) 36.7% (30.6-44.1) 0.0%	<0.0001
β2-m (at 12 months)	Normal Raised	89.8% (84.5-95.4) 20.0% (14.1-28.4)	<0.0001
LDH (at 24 months)	Normal Raised	78.6% (71.5-86.3) 21.6% (17.0-27.3)	<0.0001
Hypo-IgG	No Yes	67.3% (59.6-76.0) 40.9% (32.6-51.4)	0.1461
CD38/CD19 (>7%)	Negative Positive	67.9% (60.3-76.4) 32.5% (26.0-40.7)	<0.0001
CD38/CD19 (>30%)	Negative Positive	66.6% (59.5-74.6) 25.3% (19.5-32.7)	<0.0001
CD3	<5% 6-15% 16-30% >30%	29.0% (23.0-36.5) 52.0% (44.4-60.8) 84.5% (74.4-95.9) 91.7% (78.4-100.0)	0.0002
ZAP-70 (≥10%)	Negative Positive	69.4% (60.6-79.5) 41.1% (34.7-48.7)	0.0021
ZAP-70 (≥20%)	Negative Positive	64.4% (56.4-73.5) 45.4% (37.6-54.8)	0.0764
Morphology	Typical Atypical	62.7% (55.4-71.0) 49.3% (40.0-60.6)	0.0698
Cytogenetic aberration	del17p>20% del11>10% Trisomy 12 del13q Normal	0.0% 15.6% (11.8-20.5) 41.7% (27.9-62.3) 66.4% (57.6-76.5) 66% (54.1-80.4)	<0.0001
IGHV	Mutated Unmutated	77% (68.6-86.4) 24.8% (21-29.3)	<0.0001

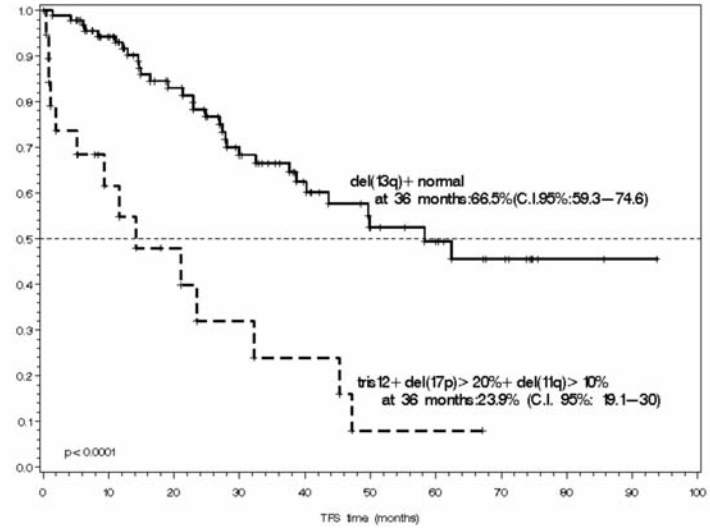
	Hazard Ratio (95% CI)	*P* value
Age	1.026 (0.984-1.07)	0.2314
Hb g/dl	0.747 (0.62-0.901)	0.0023
PLTS $\times 10^9$/L	0.993 (0.988-0.997)	0.0033
WBC $\times 10^9$/L	1.014 (1.01-1.019)	<0.0001
CLL lymphocytes $\times 10^9$/L	1.002 (1.002-1.003)	<0.0001
$CD3^+$ lymph/CLL lymph ratio	0.007 (0.000-0.109)	0.0004
CD4/CD8	1.074 (0.787-1.466)	0.6538



Online Supplementary Figure S1. TFS in CLL patients from diagnosis. TFS of 112 young CLL patients (median 45.2 months).



Online Supplementary Figure S2. TFS in CLL patients according to the CD38 expression. TFS of 29 CD38+ (>7%) and 82 CD38- CLL patients.



Online Supplementary Figure S3. 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.