SUPPLEMENTARY APPENDIX

The prognostic impact of minimal residual disease in patients with chronic lymphocytic leukemia requiring first-line therapy

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 doi:10.3324/haematol.2013.099796
Manuscript received on October 17, 2013. Manuscript accepted on December 31, 2013.
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SUPPLEMENTAL METHODS

Data collection

Data collected included age, sex, Rai and Binet stage, beta$_2$-microglobulin (B2M), date of first-line therapy, response to first-line therapy, date of second-line therapy (if there was any) and date and status at last follow-up. Biologic markers such as ZAP-70 and CD38 expression, immunoglobulin heavy chain variable region (*IGHV*) mutational status, presence of *NOTCH1, TP53* or *SF3B1* mutations; and fluorescent in-situ hybridization (FISH) analysis were also recorded.

Molecular analysis

IGHV-IGHD-IGHJ rearrangements and mutational status were assessed using leader or consensus primers for the IGHV FR1 along with appropriate IGHJ gene primers. Sequence data were aligned to IMGT databases by means of the IMGT/V-QUEST tool (http://www.imgt.org). Sequences with ≥98% of homology with the germ line were considered unmutated. For *TP53* mutation analysis, we performed direct Sanger sequencing of genomic DNA in four different PCR reactions encompassing exons 4-9.[1] *TP53* mutations were analyzed using multiple sequence alignment tools (ClustalW; http://www.ebi.ac.uk/Tools/msa/clustalw2) and wild type sequences from the IARC Database (http://www-p53.iarc.fr). Exon 34 of *NOTCH1* was amplified in two fragments that cover 97% of *NOTCH1* mutations, as previously described.[2,3,4,5] Exons 14, 15, 16 and 18 of *SF3B1* were sequenced as previously described.[2,6] In 58 patients, molecular results were extracted from whole exome sequencing analysis as previously described.[6]

MRD evaluation

Up until 2001, MRD was studied by using triple monoclonal antibody (MoAb) combinations (κ-FITC/λ-PE/CD19-PE-Cy5, CD20-FITC/CD5-PE/CD19-PE-Cy5, CD22-FITC/CD23-PE/CD19-PE-Cy5 with or without FMC7-FITC/CD79b-PE/CD19-PerCP-Cy5.5).[7,8] After 2001, quadruple MoAb combinations were used: CD20-FITC/CD79b-PE/CD19-PerCP-Cy5.5/CD5-APC, CD22-FITC/CD23-PE/CD19-PerCP-Cy5.5/CD5-APC, κ-FITC/λ-PE/CD19-PerCP-Cy5.5/CD5-APC and CD20-FITC/CD38-PE/CD19-PerCP-Cy5.5/CD5-APC.[9,10] Briefly, after incubation with the appropriate MoAb combinations, a minimum of 300,000 cells were acquired for each tube. CLL cells were identified according to their specific phenotype and the proportion of atypical cells was recorded for each combination. The MRD value in each experiment was calculated as the mean value obtained with the different combinations, excluding κ/λ antigens. Samples were considered positive when at least 30 cells with a CLL phenotype were detected in at least 2 out of 3 tubes (for triple combinations); or in 3 out of 4 tubes (for quadruple combinations). Patients who had MRD tests performed in both peripheral blood and bone marrow samples were required to have negative results in both tests to be considered as MRD negative. Since 2005, MRD was quantified according to the standardized methodology proposed by the ERIC group.[11]

Statistical analysis

Treatment-free survival (TFS) was calculated from a landmark time to the date of second line therapy, death or last follow-up. Overall survival (OS) was calculated from the same landmark time to the date of death or last follow-up. The landmark time chosen was 9 months from initiation of frontline therapy, since the average duration of CLL therapy is 6 months and most patients are evaluated 2-3 months after the last course of treatment.

Curves for TFS and OS were obtained according to the Kaplan-Meier method and the effect of different covariates was evaluated using the log-rank test. These covariates were *IGHV* mutational status (mutated *vs.* unmutated), *NOTCH1* mutations (presence *vs.* absence), *SF3B1* mutations (presence *vs.* absence), CD38 expression (higher *vs.* lower than 30%), ZAP-70 expression (higher *vs.* lower than 20%), FISH aberrations (17p deletion *vs.* 11q deletion *vs.* others), *TP53* mutations (presence *vs*. absence), B2M (above or below the upper normal range) and response to therapy. For patients who achieved MRD negativity after therapy, we calculated time to MRD relapse and time to second line therapy using Kaplan-Meier plots since there were no CLL-unrelated deaths in this cohort (i.e. there were no competing events).

Cox regression multivariate models were fitted in order to assess the independent prognostic value of those covariates that were significant by univariate analysis. The proportional hazards assumption was visually and statistically checked using Schönfeld residuals. Multiple imputations were also performed to evaluate the effect of missing data on multivariate models (AmeliaII package, R, version 3.0.1). For the purpose of multivariate models, patients were classified into those who achieved a MRD negative status *vs.* those who remained MRD positive.

SUPPLEMENTAL RESULTS

Minimal residual disease

The 4-color standardized panel described by the ERIC group was used in 51 cases,[11] a 4-color panel of 3-4 tubes in 22 patients, and a 3-color panel of 3 tubes in 23 patients. MRD was measured in both peripheral blood and bone marrow in 79 patients, in peripheral blood only in 12 patients, and in bone marrow only 5 patients. The median number of cells acquired in samples with MRD < 0.1% was 1,309,013 (range: 281,076-1,500,000 cells) in peripheral blood and 1,500,000 (range: 472857-1,500,000 cells) in bone marrow. In 22 samples with sensitivity > 10^{-4}, the MRD value was greater than the assay's sensitivity and these samples were considered as MRD positive. Supplemental Table 2 shows the distribution of samples according to MRD sensitivity.

There were five patients whose MRD test did not achieve the required sensitivity (<10^{-4}) without evidence of residual CLL cells. In three of these patients, the assay sensitivity ranged from 1.5 x10^{-4} to 2.8 x10^{-4}, but the following MRD test did achieve a sensitivity <10^{-4} and the result was negative. In the remaining two patients the sensitivity of the assay was borderline (1.1 x10^{-4} and 1.2 x10^{-4}, respectively), but none of them had any more MRD tests. All five patients were considered to have MRD negative disease.

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SUPPLEMENTAL TABLES

Supplemental Table 1: List of *TP53*, *NOTCH1* and *SF3B1* mutations detected in our cohort

TP53	n = 22	**NOTCH1**	n = 39	**SF3B1**	n = 28
R248W/Q	3	P2515fs*4	35	K700E/I	9
R158G	2	L2483fs*1	2	K666E/T	5
R209fs*6	2	Q2504*	1	G740D/E	2
T155I	2	Q2405*	1	N626Y	2
Q136E	1			R625H	2
S215G	1			K741N	2
G244D	1			G742D	2
T284*	1			V701F	1
M237K	1			T663I	1
H175R	1			D894G	1
Y234*	1			H662D	1
I254M	1				
P278S	1				
V274F	1				
W91X	1				
S315F	1				
c.248_249ins8	1				

Supplemental Table 2: Distribution of samples according to MRD sensitivity

Sensitivity	**PB**	**BM**	**PB+BM**
$< 10^{-4}$	5	3	61
$> 10^{-4}$	2		3
$> 10^{-4}$, but MRD > sensitivity	5	2	15