Clinical profile associated with infections in patients with chronic lymphocytic leukemia. Protective role of immunoglobulin replacement therapy

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SUPPLEMENTARY METHODS

Prognostic factors were evaluated on fresh sample or on purified frozen CLL cells harvested in DMSO, collected before chemo-immunotherapy according to recent guidelines[1].

Fluorescent in situ hybridization
Fluorescent *in situ* hybridization (FISH) was performed on standard cytogenetic preparations from peripheral blood. The slides were hybridized with the multicolor probe sets LSI p53/LSI ATM, LSI D13S319/LSI 13q34/ CEP12 and RP11-177O8 (Vysis-Abbott, Des Plaines, IL, USA) according to the manufacturer's protocol. Three hundred interphase nuclei were analyzed for each probe. Accordingly with the literature, cut-off for positive values (mean of normal control ±3 standard deviation) was 4% for centromere 12 trisomy, and 10% for deletion of 11q22.3, 13q14.3 and 17p13.1 [2,3].

Immune globulin heavy chain variable region mutation
To perform IGHV studies, RNA was extracted from $2x10^6$ B cells using the RNeasy™ Total RNA kit (Qiagen) and reverse transcribed using the SuperScript™ Preamplification System for first-strand cDNA synthesis (Life Technologies, Inc.). The B-CLL cell VH gene family was assigned as previously described [3] using a sense VH family-specific framework region (FR) primer in conjunction with the appropriate antisense CH primer. VH gene sequences were determined by amplifying 5µl of the original cDNA using the appropriate VH leader and CH primers. PCR products were sequenced directly after purification with Wizard PCR Preps (Promega, Madison, WI) using an automated genetic analyzer (3130 ABI Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using IMGT/VQUEST and BLAST software [4] to detect VDJ junction. Sequences homology ≤98, from the corresponding germline gene, were considered mutated, as opposite to unmutated cases [3,5].

CD38 expression
Analyses of CD38 expression on B-CLL cells was carried out by incubating whole blood with 5µl of the following antibodies: anti-CD5 FITC (BD Biosciences), anti-CD38 PE (BD Biosciences), and anti-CD19 RPECy5 (BD Biosciences), for 20 minutes and at least 100,000 events were counted. Each sample was run with the appropriate isotype control antibodies to define the negatively stained cells. The percentage of $CD38^+$ cells was defined as the percentage of $CD19^+/CD5^+$ that were $CD38^+$. The threshold for CD38 expression was set at 30%; values ≥30% were defined as $CD38^+$ and <30% as $CD38^-$ [3,6]

ZAP70 expression
Cytoplasmic ZAP-70 expression was determined by flow cytometry. Permeabilized cells were analysed with the anti-ZAP-70 antibody Alexa Fuor488 (Caltag), CD3-phycoerythrin (PE), CD56-PE (BD Biosciences), CD 19-peridinin chlorophyll protein-cytochrome 5,5 (Caltag) and CD 5 APC (BD Biosciences). After appropriate lymphocyte gating, cytoplasmic ZAP-70 expression was determined in $CD19^+$ $CD5^+$ B-CLL cells. The threshold level for ZAP-70 was set at 20% [3,7].

Mutational analysis
The mutation hot spots of the *TP53* (exons 4-9, including splicing sites), *NOTCH1* (exon 34; including splicing sites), *SF3B1* (exons 14, 15, 16, 18, including splice sites), *MYD88* (exons 3, 5, including splicing sites), and *BIRC3* (exons 6-9, including splicing sites) genes were analyzed by PCR amplification and DNA direct sequencing on DMSO frozed CLL cells collected at presentation or at disease progression by Rossi D et al [8].

Immunoglobulin replacement therapy strategies

As recently reported[9], in our Unit replacement therapy with either intravenous (IVIG, 300 mg/kg/every three weeks) or subcutaneous immunoglobulins (SCIG, 75 mg/Kg/week) was started in subjects showing hypogammaglobulinemia (IgG <600 mg/dl) and i) complaining serious non neutropenic infectious events, or ii) when an increase of the incidence of non neutropenic infections requiring antibiotic therapy was detected (more than 2 episodes in 12 months).

Statistical analysis

Categorical variables were compared by Chi-square test, while continuous variables were compared by Mann-Whiney or Kruskal-Wallis test, when appropriated. Overall survival (OS) was calculated starting from the date of initial presentation to death for any cause (event) or last known follow-up (censored). Survival analyses were performed by Kaplan-Meier method and Log-rank test was used to compare overall survival curves between groups. Cox regression model was employed to estimate hazard ratios; the proportional hazard assumption was tested in all cox models. The stability of Cox model was internally validated using bootstrap .632 method with B=706. The predictive accuracy and error of the cox models were evaluated by the Harrel's concordance index (c-index) and by 1 - c-index, corrected for optimism and estimated by .632 bootstrap method, respectively. The predictive accuracy is the probability of concordance between predicted and observed survival. A c-index of 0.5 indicates the outcomes are completely random, whereas a c-index of 1 indicates that the model is a perfect predictor. Covariables analyzed in multivariate analysis for OS were MI event, age>65 years, male gender, Binet C, Rai III-IV, U-IGHV, 17p deletion, 11q deletion, CD38+ and ZAP70+. To detect the possible level of protection related to the levels of the different IG isotypes a receiver-operating characteristic (ROC) curve were used. p values ≥0.05 were considered as not significant. Statistical analysis was performed with R (an open source statistical package downloadable from http://www.r-project.org).

SUPPLEMENTARY RESULTS

Data of previous need for chemotherapy and combined antibody deficiency were combined in a unique cox-model, showing the shorter time to major infection for patients with both the abovementioned markers. This model was internally validated by bootstrap .632 method, showing a predictive accuracy, determined by c-index, of 0.68 and a predictive error of 0.34.

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Supplementary Table

Table SI. Median months to infection and hazard ratio by univariate and multivariate analysis, respectively.

	univariate			multivariate		
	median	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
AGE						
<65 years	271	229-nr	0.0341	1.00	-	0.0124
≥65 years	239	188-240		1.82	1.13-2.94	
GENDER						
Female	356	261-nr	0.1629	1.00	-	0.7087
Male	239	218-251		1.24	040-3.81	
STAGE						
0-I-II	356	233-nr	0.0002	1.00	-	0.0001
III-IV	217	160-248		2.35	1.50-3.71	
IGHV						
M-IGHV	356	229-nr	<0.0001	1.00	-	<0.0001
U-IGHV	217	138-232		3.61	2.10-6.18	
FISH						
13q/N/+12	nr	204-nr	<0.0001	1.00	-	<0.0001
11q and/or 17p	146	76-228		3.58	2.16-5.93	
CD38						
<30%	250	219-357	0.0254	1.00	-	0.0160
≥30%	227	147-nr		1.81	1.11-2.94	
ZAP70						
<20%	247	206-nr	0.8967	1.00	-	0.8740
≥20%	232	219-358		1.04	0.64-1.69	
TP53/NOTCH1/BIRC3/SF3B1						
Wild-type	nr	38-nr	0.9007	1.00	-	0.7451
Mutated	250	49-245		0.82	0.25-2.69	

95% CI= 95% confidential interval, nr=not reached.

Supplementary Figure

Figure S1. Left panel (a) shows Kaplan Meier analysis of overall survival of patient with (PwI, dashed line) and without a history of major infection (PwoI, continuous line). The median OS was 196 months for PwI but was not reached for PwoI (Log-rank test, p<0.0001). The right panel (b) report hazard ratio of all the analyzed covaribles: age>65 year 5.63 (95% CI 3.52-0.01, p<0.0001), U-IGHV 4.56 (95% CI 2.79-7.45, p<0.0001), del 17p 3.32 (95% CI 1.73-6.38, p=0.0001), del 11q 2.68 (95% CI 1.40-5.13, p=0,0019), Major Infection 2.34 (95% CI 1.51-3.62, p<0.0001), Binet C 2.32 (95% CI 1.26-4.27, p=0.0055), Rai III-IV 2.29 (95% CI 1.25-4.20, p=0.0061), male gender 1.89 (95% CI 1.21-2.95, p=0.0042), CD38+ 1.73 (95% CI 1.10-2.73, p=0.0165), ZAP70+ 1.42 (95% CI 0.06-2.29, p=0.13766).