Modulated expression of adhesion, migration and activation molecules may predict the degree of response in chronic lymphocytic leukemia patients treated with ibrutinib plus rituximab

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Supplementary Methods

Patients

The study was carried out on a series of 119 CLL patients enrolled in the GIMEMA LLC1114 phase II front-line IR trial (clinicaltrials.gov identifier: NCT02232386) between March 2015 and April 2017. Inclusion criteria comprised previously untreated CLL patients with indication for treatment in accordance with the 2008 International Workshop on Chronic Lymphocytic Leukemia (IWCLL) guidelines (*Hallek M et al, 2017*). Treatment consisted of ibrutinib (420 mg/die) continuously daily in combination with rituximab (375 mg/m^2/week) for weeks 1 to 4 (cycle 1 = 28 days); rituximab was then given once every 4 weeks until cycle 6. All patients gave their informed consent in accordance with the Declaration of Helsinki. The study was approved by the Ethic Committee of the Sapienza University. The biologic work-up included the IGHV gene mutational status, ZAP-70, CD38 and CD49d evaluation by flow cytometry, fluorescence in situ hybridization (FISH) analysis for deletions at the 11q22-23, 13q14, 6q21, 17p13 regions and trisomy 12, as well as *TP53* sequencing. Lymphocytosis after the start of ibrutinib was defined as a ≥50% increase in absolute lymphocyte counts (ALC) compared to baseline and an absolute cell count >5x10^9/L (*Burger JA et al, 2015*). The characteristics of CLL patients included in the study are detailed in Supplementary Table 1.

Flow cytometry

PB CLL samples were collected prior to treatment (D0), on day 14 (D14) and 8 months after the start of IR treatment (T8m). For flow cytometric (FC) evaluation, fresh cells upon arrival were stained with an appropriate volume of monoclonal antibodies (mAbs) (from Becton Dickinson, San Jose, CA) in different combinations, acquired using the FACSCanto II flow cytometer (Becton Dickinson), and analyzed using the PAINT-A-GATE and FACSDIVA softwares (Becton Dickinson) through specific gating strategies. CLL cells were immunophenotyped by using FITC-, PE-, PerCP-, PerCP-Cy5.5-, APC, APC-Cy7-, Horizon V450- and Horizon V500- labelled mAbs against CD45, CD3, CD5, CD19, CD20, CD200, CD22, CD23, FMC-7, CD79-b, CD27, CD10, Igκ and Igλ; the mean fluorescent intensity (MFI) of CD11a, CD18, CD38, CD40, CD43, CD44, CD49d, CD62L, CD69, CD80, CD81, CD86, CD154, CD184 and CD185 on leukemic B cells was assessed. Cell surface expression was quantified as the MFI of values obtained with specific mAbs compared with values given by the isotype controls. The cut-off value for CD38 and CD49d cell positivity was determined at 20%. The two groups with different CD20 expressions were based on the median value of the geometric MFI of CD20. The average percentage of lymphocytes that were CLL cells was 89.7%±10.4 (range 30-98%) at baseline and 82.0%±22.1 (range 6-98%) on D14.

For FC MRD evaluation at T8m, PB samples were stained and analyzed using the 8-color mAb combination against CD45, CD19, CD20, CD5, CD38, CD81, CD43 and CD3 together with Ig evaluation. The presence of MRD was reported as the percentage of pathologic cells within the total leucocyte population with a sensitivity limit equal to 0.01%.

Instrument quality controls were performed by means of CST beads (Becton Dickinson, BD) according to the manufacturer's instructions. Photomultiplier settings were established by taking advantage of BD OneFlow Setup Beads and the same beads were acquired before each sample analysis to ensure the stability of the system, thus allowing a robust and correct comparison of MFI data generated over time.

Statistics

Statistical analyses were performed using the Student t-test and χ^2/Fisher exact test. Statistical significance was defined as a P value <0.05.

Supplementary Table 1. Baseline demographic, biological and clinical characteristics of the patients included in the study.

Characteristics	Total (n=119)
Age (yr)	72 (37-87)
Sex (no.)	
Male	74
Female	45
IGHV status (no.)	
Mutated	51
Unmutated	66
N.A.	2
TP53 status (no.)	
Mutated	21
Wild-type	98
Cytogenetics, FISH (no.)	
del17p	14
del11q	20
tris12	21
del13q14	29
normal karyotype	33
N.E.	2
White blood cell count ($x10^9$/L)	
D0	67.9 (3.2-216.4)
D14	65.8 (2.4-299.4)
Absolute lymphocyte count ($x10^9$/L)	
D0	51.5 (1.4-148.2)
D14	51.6 (0.9-206.3)

Data are expressed as no. or median (range). N.E.: not evaluable