miR-125b and miR-532-3p predict the efficiency of rituximab-mediated lymphodepletion in chronic lymphocytic leukemia patients. A French Innovative Leukemia Organization study

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Gene expression analysis

RNA extraction

Blood was collected before treatment in PAXgene Blood RNA Tubes according to the instructions in the PAXgene Blood RNA Tube Product Circular. Total RNAs, including small RNAs, were extracted using the PAXgene Blood miRNA Kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instruction with minor modifications related to the B cell amount. Blood samples were divided as often as necessary to load a maximum of 40G/L lymphocytes per column.

RNA concentration and purity were assessed using the NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, USA). RNA quality was assessed using the 2100 Bioanalyzer assay (Agilent Technologies, Les Ulis, France), and according to the criteria of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments MIQE guidelines, only samples with a RIN>8 were used.[1]

miRNA TaqMan Low-Density Array

Two groups of patients were analyzed with the TaqMan Low-Density Array (TLDA) technology according to the lymphocyte concentration at Day 0 (D0). One group called "Low" presented lymphocyte count inferior to quartile 1 (11.67 G/L) of the lymphocyte concentration at D0 for all patients: to 4.6 G/L to 10.7 G/L (n=5). The other group called "High" displayed lymphocyte count superior to quartile 3 (93.93G/L) of the lymphocyte concentration at D0 for all patients: to 97 G/L to 223 G/L at D0 (n=5).

Total RNAs (70 ng) were converted into cDNAs using Megaplex™ RT Primers (human pool A v2.1) and TaqMan® MicroRNA Reverse Transcription kit. The RT pool detected 377 miRNAs. A pre-amplification step using Megaplex™ PreAmp Primers (human pool A v2.1) and TaqMan® PreAmp Master Mix was performed. MicroRNA profiling was achieved using the TaqMan® Human MicroRNA Array Cards A v2.0 and TaqMan® Fast Advanced Master Mix. The 384-well format TLDAs were run on a ViiA 7 real-time PCR system (Thermo Fisher Scientific, Waltham, USA). All reagents were supplied by Thermo Fisher Scientific, Waltham, USA.

miRNA array analysis

RT-qPCR raw data were analyzed using SDS 2.3 and RQ Manager Software (Thermo Fisher Scientific, Waltham, USA). Each miRNA for each sample was normalized to the mean threshold cycle (Ct) value of all expressed miRNAs and RNU48. Relative miRNA expression was calculated using the comparative Ct method. Using a fold change (FC) ± 1.5 ($P \leq 0.05$), and a two-dimensional hierarchical clustering analysis, we selected a set of miRNAs correlated with the lymphocyte concentration at D0.

miRNA validation

MicroRNA validation consisted in a RT-qPCR on the Dense-FCR arm of the protocol. We performed a multiplex RT and pre-amplification from 60 ng of RNA, using TaqMan® MicroRNA Reverse Transcription kit and TaqMan® PreAmp Master Mix, respectively. PCR for each miRNA was performed for each patient using TaqMan® Fast Advanced Master Mix. The 96-well format plates were run on a ViiA 7 real-time PCR system (ThermoFisher Scientific, Waltham, USA). All reagents were supplied by Thermo Fisher Scientific, Waltham, USA. RNU48 was used for normalization to obtain relative miRNA expression.

mRNA quantification

MS4A1 (Hs00544819_m1), MS4A3 (Hs00960994_m1) and MS4A7 (Hs009600225_m1) mRNA expression levels were quantified using the Taqman gene expression assays (Thermo Fisher Scientific,

Waltham, USA). GAPDH (4333764F) was used as endogenous control for mRNA data normalization. Relative mRNA expression was calculated using the comparative Ct method.

***FCGR3A* genotyping**

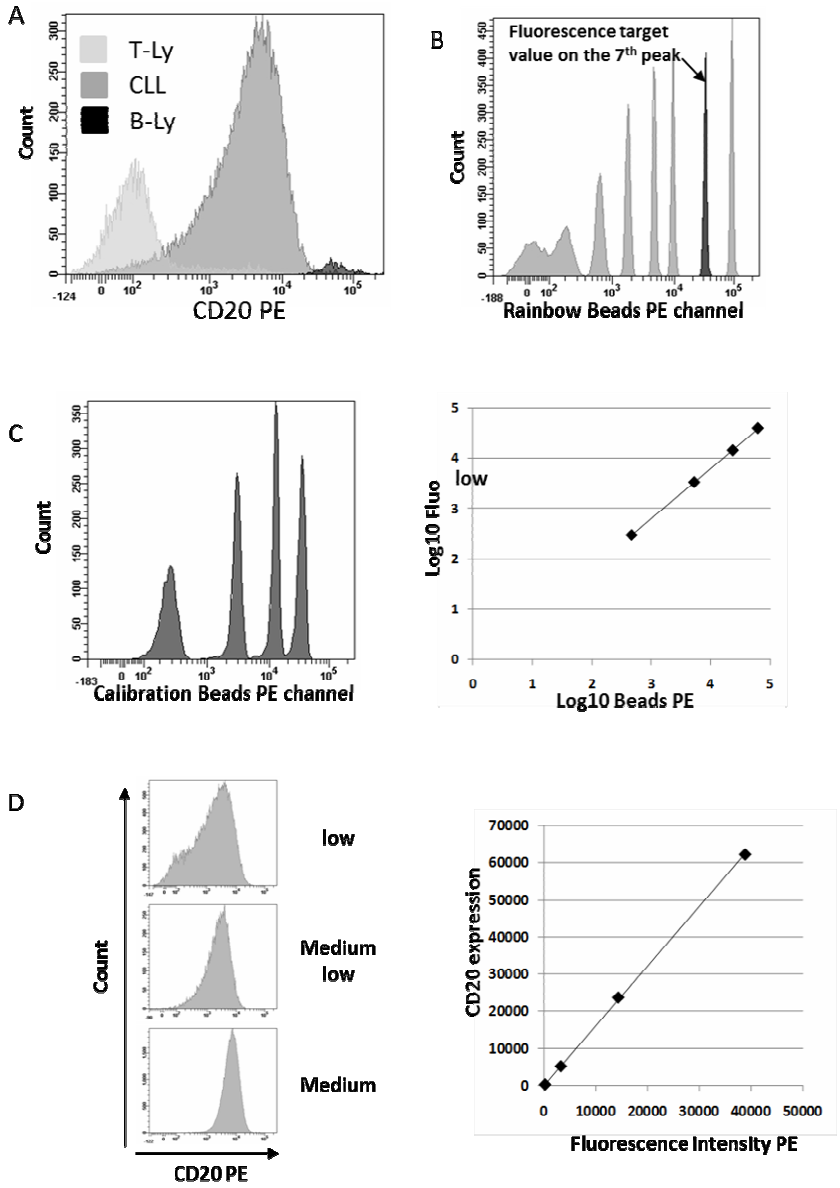
Nested PCR was performed as previously described, with slight modifications.[2-4] Briefly, two *FCGR3A*-specific primers (5'-ATATTTACAGAATGGCACAGG-3' and 5'-GACTTGGTACCCAGGTTGAA-3') (Eurobio, Les Ulis, France) were used to amplify a 1.2-kb fragment containing the polymorphic site. The initial PCR assay was performed with 1.25 µg genomic DNA, 200 ng of each primer, 200 µM of each deoxyribonucleoside triphosphate (dNTP) (MBI Fermentas, Vilnius, Lithuania) and 1 U Taq DNA polymerase (Promega, Charbonnière, France). This first PCR consisted of 10 min at 95°C, then 35 cycles (each consisting in 95°C for 1 min, 57°C for 1.5 min and 72°C for 1.5 min), and 8 min at 72°C to achieve complete extension. The second PCR used primers (5'-ATCAGATTCGATCCTACTTCTGCAGGGG-GCAT-3' and 5'-ACGTGCTGAGCTTGAGTGATGGTGATGTTC-AC-3') (Eurobio, Les Ulis, France) amplifying a 94-bp fragment and creating an *Nla*III restriction site only in the *FCGR3A*-158V allele. This nested PCR was performed with 1 µL of amplified DNA, 150 ng of each primer, 200 µM of each dNTP, and 1 U of Taq DNA polymerase. The first cycle consisted of 5 min at 95°C, then 35 cycles (each consisting in 95°C for 1 min, 64°C for 1 min and 72°C for 1 min), and 9.5 min at 72°C to complete extension. The amplified DNA (10 µL) was then digested with 10 U *Nla*III (New England Biolabs, Hitchin, England) at 37°C for 12h and separated by electrophoresis on 8% polyacrylamide gel (37:1 ratio acrylamide/bis-acrylamide). After staining with ethidium bromide, PCR products were visualized under UV transillumination. Only one undigested band (94 bp) was visible for homozygous *FCGR3A*-158F individuals, three bands (94, 61 and 33 bp) were seen in heterozygous individuals, whereas only two digested bands (61 and 33 bp) were obtained for homozygous *FCGR3A*-158V individuals.

CD20 expression cell detection

CD20 expression was quantified using the commercial kit QuantiBRITETM CD20PE (Ratio 1:1) according to manufacturer's recommendations (BD Biosciences, Le Pont-de-Claix, France).

Initial cytometer setup was performed to allow the study of CD20 expression on T-lymphocytes as negative control, normal B-cells as positive control and CLL cells. Then fluorescence target values were determined for PE channel using 8-peak Rainbow bead calibration particles (Spherotech, Lake Forest IL, USA) and a calibration curve for CD20 QuantiBRITETM assay was established based upon these settings. Before making any new calibration curve, the cytometer setup was adjusted to reproduce the initial settings using the same lot of 8-peak Rainbow bead calibration particles.

Supplementary figure shows representative cases of CD20 expression on CLL cells. By using a calibration curve, the measure of CD20 fluorescence intensity on CLL cells allows calculating the number of equivalent CD20 molecules present at the cell surface.



Supplementary figure: Study of CD20 expression on CLL cells using QuantiBRITE™ CD20PE commercial kit.

Panel A shows the initial setting for the study of CD20 expression on T-lymphocytes , normal B-cells ■, and CLL cells . Panel B shows the determination of the fluorescence target value for PE channel using Rainbow beads. Panel C shows the use of the calibration beads to measure the fluorescence intensity of the beads and construct the calibration curve. Panel D shows representative cases of CD20 labeling on CLL cells. The determination of the fluorescence intensity and the use of an antiCD20 reagent certified with a PE to monoclonal antibody ratio of 1:1, allows calculating (with the calibration curve of panel C) the number of equivalent CD20 molecules present at the cell surface.

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