Gene expression profiling of chronic lymphocytic leukemia can discriminate cases with stable disease and mutated Ig genes from those with progressive disease and unmutated Ig genes

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TO THE EDITOR

The demonstration that the clinical behaviour is related to the mutational status of immunoglobulin (Ig) genes[1] suggested two independent cellular origins for this disease. Two previous microarray studies, however, clearly demonstrated that CLL has a unique transcriptional profile, irrespective of its mutational status.[2,3] Moreover, although a relatively small number of genes were differentially expressed between Ig-mutated and Ig-unmutated CLLs, unsupervised analyses failed to segregate these two forms. The Ig mutational status is not, however, the sole determinant of clinical outcome. We recently showed in 145 CLL cases, that Binet staging still retained its independent prognostic value since four distinct groups could be individualized according to Binet's staging and mutational status of Ig genes: (1) Ig-mutated stage A with a 75% 12-year survival expectancy, (2) Ig-mutated stage B and C patients with a median overall survival (OS) of 120 months, (3) Ig-unmutated stage A (median OS of 97 months) and (4) Ig-unmutated stages B and C (median OS of 78 months).[4] These results suggest the existence of other pathogenic pathways underlying such differences in addition to the Ig mutational status. With these considerations in mind, we rechallenged the two distinct diseases hypothesis by analyzing the transcriptional profiles of patients clustering at the extremes of the CLL spectrum, that is, stable mutated (SM) stage A forms *vs* progressive unmutated (PU) stage B/C ones.

Peripheral blood samples from 18 patients with CLL were analyzed after informed consent, according to our institutional regulations. Nine patients had stage A, stable disease with at least 5 years of follow-up without treatment or disease progression, and with mutated Ig (SM group). Nine had progressive stage B or C disease, which had been treated according to the French Cooperative Group on CLL's protocols, and expressed unmutated Ig (PU group). Clinical and laboratory data are shown in Table S1 on the *Leukemia* website (Supplementary Information). All leukemic cells were collected prior to chemotherapy. Peripheral blood mononuclear cells were separated by density gradient centrifugation (Ficoll-Paque Plus, Amersham, Saclay, France), and stored in liquid nitrogen. Upon thawing, leukemic cells viabilility was assessed by trypan blue staining, and further processed only if it was $>80\%$. They were then purified by negative magnetic selection using anti-CD3, anti-CD14, anti-CD16 and anti-CD56 monoclonal antibodies (Dynal, Oslo, Norway). B-cell purity was evaluated by flow cytometry to be $>98\%$. Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA, USA) including a DNAse treatment step. Integrity of the RNA was assessed by capillary electrophoresis (Agilent Technologies, Massy, France). cRNA was prepared according to the standard Affymetrix protocol and $10\,\mu g$ hybridized to GeneChip HG-U133A oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA). Expression values were determined using Affymetrix MAS 5.0 software. The gene expression data are available on our website: http://amazonia.montp.inserm.fr/downloads.php.

From the 22 283 probe sets present on the U133A array, 11 434 had absolute calls defined as 'present' by the GeneChip software in at least three samples among the 18 tested, and were further retained for analysis. All expression levels values below a threshold arbitrarily chosen as the quarter of the median value across all samples were floored to this default value ($=10$). The expression level of each gene was first log-transformed and median-centered before being processed. Hierarchical clustering was performed using Cluster and Treeview software packages.[5] Initial unsupervised hierarchical clustering failed to distinguish SM cases from PU cases (data not shown). This was probably due to the low proportion of genes differentially expressed in the two groups (see below). However, restricting this analysis to the 840 probe sets which showed the highest variation in expression levels among all samples resulted in a clear segregation of CLLs in two clusters: all but one SM case (CLL6-SM) were correctly classified according to their clinical stage and mutational status (Figure 1 and Figure S1 in Supplementary Information).

To confirm these results, we used principal component analysis as a second unsupervised algorithm. This was performed on log-transformed and median-centered data from the 4150 genes with the highest variation coefficient among the 18 samples. The first two principal components, which represented 22% of the total variance, identified two clusters, which corresponded to the clinical groups except for two cases (CLL6-SM and CLL11-PU) (Figure S2, supplemental data). Thus, taking into account that we selected cases representing both ends of the disease spectrum and restricted the analysis to the 840 most differentially expressed genes, both unsupervised methods could distinguish the majority of SM- from PU-CLLs by their pattern of gene expression.

Supervised analysis was performed using the Mann–Whitney nonparametric statistical and calculations were performed with the SPSS software (SPSS Inc, Chicago IL, USA). Of the 11434 genes, 733 were differentially expressed among SM and PU groups (P-value <0.05). However, a >2-fold change was found for only 84 of these after exclusion of Ig genes (Tables S2 and S3, Supplemental data). For the majority of them, the fold change was within a 2–3 range. It was above three for 32 genes, and above four for only 14 genes. PU-CLL cases overexpressed 43 genes including some previously recognized such as *BCL7A*, *LPL* (lipoprotein lipase), *AKAP12* (gravin), *CRY1*, *CLECSF2*, *DMD* (dystrophin) and *ZAP-70*. In contrast with previous studies, an equivalent number of genes ($n=41$) were also overexpressed in SM-CLL cases, the most prominent of which were *MYL9*, *NRIP1*, *FCGBP*, *SPRY2*, *TCF7* and *ADAM29*. Table 1 includes the 26 genes that displayed a >3-fold change and a P-value $\leqslant 0.01$, as well as two other genes (*WNT3*, *SPAP1*) which had a slightly lower fold change (2,5 and 2,3,

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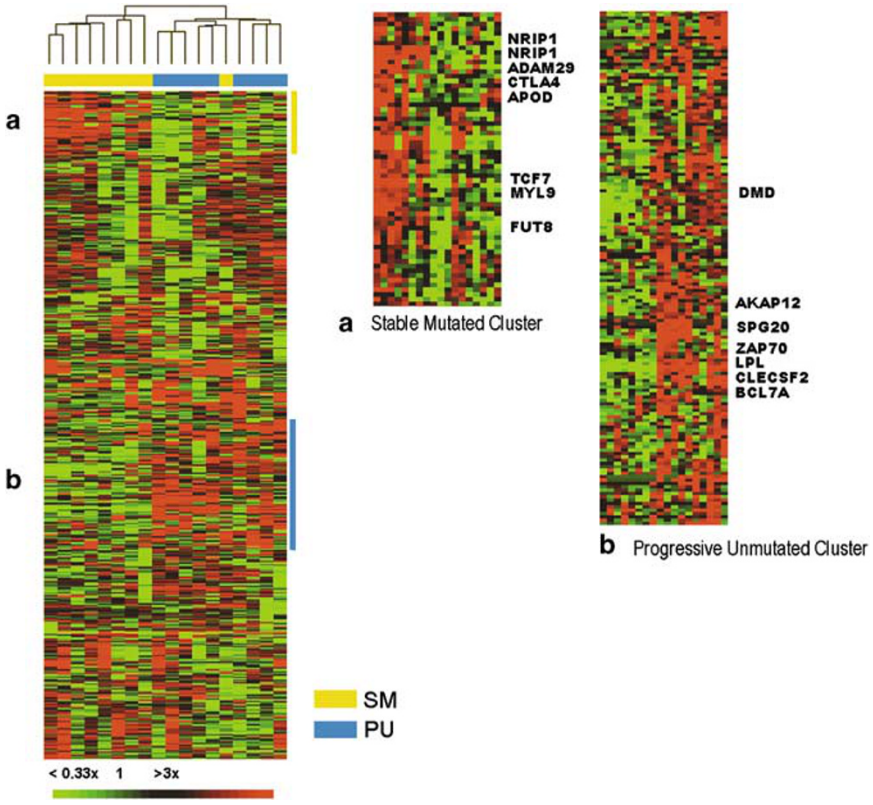

Figure 1 Unsupervised clustering of 18 cases of CLL. Hierarchical clustering of 840 genes having the highest variation in their expression level is shown in the left panel. The central panel corresponds to the genes overexpressed in SM-CLLs (a), while the right hand one shows those overexpressed in PU-CLLs (b). Expression value for each gene is represented relative to its median value across all samples, according to a color scale in which red and green indicate higher and lower expression levels, respectively. The cluster dendrogram shows the delineation of SM cases (yellow bar) from PU cases (blue bar) except for one SM-CLL, which segregates with the PU group. The sample and gene names are depicted on Figure S1 in Supplementary Information.

respectively) but were involved in pathways such as *TCF7* regulation and BCR signalling.

Since the number of patients we analyzed was small, this may have led to false identification of discriminating genes, as a result of chance only. Thus, a statistical permutation permutation test was used to estimate how many genes could be expected to appear differentially expressed from random class distinction. This was made with the R environment (http://www.r-project.org). We performed 100 permutations and determined that 1.47 out of 11 434 genes would have a ratio >3 and a *P*-value $\leqslant 0.01$ in Mann–Whitney analysis by chance only.

We verified the relative expression of 18 genes by quantitative reverse-transcription polymerase chain reaction (RQ-PCR), including 16 of the 26 with a >3-fold change and a *P*-value $\leqslant 0.01$, and two other genes (*WNT3, SPAP1*). Experiments were carried out on the same purified leukemic RNAs used for microarray hybridization, for 16 of the 18 patients for whom material was still available. Of these genes, 10 (*AKAP12*, *ADAM29*, *BCL7A*, *CLECSF2*, *FCGBP*, *FLJ10884*, *FUT8*, *LPL*, *TCF7*, *WNT3*) were quantified using Assay-on-Demand Gene Expression Probes and TaqMan reagents on an ABI Prism 7000 Sequence Detector System (all supplied by Applied Biosystems, Les Ulis, France). The other eight genes (*APOD*, *SPG20*, *MYL9*, *NRIP1*, *SPAP1*, *SPRY2*, *TGFBR3*, *ZAP-70*) were quantified by the SYBR Green I method on the Light Cycler System (Roche Molecular Biochemicals, Mannheim, Germany). Primers used in the latter cases were designed with Gene Runner software (Hastings Software, Colorado, USA) and their sequences are depicted in Table S4 (Supplementary Information). All PCR reactions were performed on cDNA from 100 ng of total RNA and done in duplicate. The housekeeping beta-glucuronidase (*GUS*) gene served to normalize quantitative data. Standard curves were established following serial sample dilutions to ensure that amplification efficiency within each cycle was close to a factor 2 (data not shown). The differential expression of all of tested genes was confirmed, though a higher fold change was generally found in RQ-PCR when compared to microarray experiments (Table 1). Figure S3 (supplemental data) depicts the distribution of patients according to differential expression of six of these genes, including *ZAP-70,* which displayed the best delineation of the two groups of CLLs. As previously reported, *ZAP-70* expression clearly predominated among PU cases (eight out of nine). However, a higher fold change ratio between PU and SM cases was found for *LPL*, *FLJ10884*, and *SPG20*. In addition, two previously unrecognized discriminating genes, *ADAM29* and *NRIP1*, strongly distinguished SM (both overexpressed in six of seven cases) from PU cases (both underexpressed in eight of nine cases).

In agreement with previous reports[2,3] our results demonstrate that less than 1% of transcribed genes in CLL B-cells were significantly and differentially expressed in SM and PU patients, thus supporting the single disease hypothesis. However, in contrast with these previous studies, two independent unsupervised analytical methods succeeded in distinguishing indolent from aggressive forms of the disease. This could be accounted

Table 1 Differentially expressed genes in PU- and SM-CLLs

Genbank accession no.	*Gene*	*Microarray fold change*	*P-value*	*RQ-PCR fold change*	*P-value*
Overexpressed in PU-CLLs					
X89984	*BCL7A*	9.9	0.01	16.0	0.005
M15856	*LPL*	7.7	0.0008	97.0	0.002
AY123329	*SPG20*	6.4	0.0005	14.9	0.0001
AF243495	*EFA6R*	5.4	0.003	ND	
AK001973	*FLJ10884*	4.5	0.002	168.9	0.001
U81607	*AKAP12*	4.3	0.00004	12.1	0.005
D83702	*CRY1*	4.1	0.01	ND	
X96719	*CLECSF2*	4.1	0.002	10.5	0.005
Z36715	*ELK3*	3.6	0.004	ND	
M18533	*DMD*	3.5	0.01	ND	
BC045729	*Unknown*	3.4	0.0005	ND	
L07594	*TGFBR3*	3.3	0.002	4.3	0.02
BC039039	*ZAP-70*	3.2	0.0008	4.6	0.007
AB056059	*MAGE1*	3.1	0.01	ND	
Overexpressed in SM-CLLs					
J02854	*MYL9*	15.1	0.01	8.0	0.03
X84373	*NRIP1*	9.4	0.0005	5.6	0.0003
D84239	*FCGBP*	7.8	0.003	12.1	0.005
X59871	*TCF7*	4.5	0.0008	7.5	0.005
J02611	*APOD*	4.0	0.006	6.1	0.04
AF134708	*ADAM29*	4.0	0.008	21.1	0.003
AF039843	*SPRY2*	3.8	0.01	8.0	0.02
Y17979	*FUT8*	3.8	0.0005	4.3	0.02
AF414120	*CTLA4*	3.5	0.006	ND	
AL832961	*PTK2*	3.4	0.006	ND	
AK074062	*FLJ00133*	3.4	0.006	ND	
U92032	*HLA-DQB1*	3.2	0.01	ND	
AB067628	*WNT3*	2.5	0.01	34.3	0.02
AY043465	*SPAP1*	2.3	0.002	2.3	0.05

ND, not done.

for by the different patient inclusion criteria, the cell purification procedure (negative *vs* positive selection), and the fact that we used a more recent version of the Affymetrix DNA chip, which includes a higher number of gene probes than that employed in previous reports. The same explanations may apply to the fact that the majority of the genes that we identified as differentially expressed have not been previously reported by studies comparing CLL upon their Ig mutational status. Thus, only seven genes were in common with those described in Klein's report,[2] six with that of Rosenwald *et al*.[3] Our data suggest that CLL may be considered as a single disease with at least two variants that differ in their clinical course and Ig mutational status. It is clear, however, that this view is a simplification and that reality is more complex. For instance two patients in this series clustered in the opposite group. This indicates that other factors than the clinical stage and Ig mutational status, such as chromosome abnormalities for instance, may impact on the global gene expression profile. Haslinger *et al*[6] reported recently that gene expression profiles of B-CLL depend on genomic alterations. Unfortunately, the number of cases with distinct genetic abnormalities in our series was not sufficient to draw firm conclusions in this direction.

The mutational status of Ig is one of the most robust biological prognostic indicators in CLL. However, defining Ig gene sequence is not suitable for routine purposes. These considerations have made finding a surrogate for Ig mutational status in CLL an important priority. Following microarray studies reporting that *ZAP-70* overexpression in Ig-unmutated CLLs,[3] its detection by a simple and convenient multiparameter flow-cytometric test has recently been shown to be highly correlated to the presence of an Ig unmutated status.[7] Our microarray data, confirmed by RQ-PCR studies, show that other genes such as *LPL*, *FLJ10884*, *SPG20*, might be as discriminative as *ZAP-70*. In addition, *ADAM29* and *NRIP1* are potentially good predictors for SM patients. We have very recenly evaluated the capacity of some of these genes to predict clinical outcome in an independent series of 127 CLL patients.[8] Our data show that the quantification of *LPL* and *ADAM29* mRNAs constitutes a reliable surrogate of the Ig mutational profile and provides strong prognosis information Finally, we tried to correlate possible functions and interactions for these differentially expressed genes, according to a literature search. We focused our attention on the 28 genes which had a >3-fold change ratio with a $P \leqslant 0.01$ and/or whose differential expression had been validated by RQ-PCR. As a result of this relatively limited set of genes, we attempted to integrate their physiological properties into a schematic representation (Figure S4 in Supplementary Information). The main emerging features are the role of the B-cell receptor (BCR) activation pathway, cytoskeletal regulation and interactions with the extracellular matrix. Although these considerations are so far hypothetical, they point out new directions for further functional studies.

In summary, our study shows that gene expression profiling can differentiate SM- from PU-CLLs. It identified new genes which expression level monitoring can serve as prognostic indicators. It also points out some of the potential pathways involved in the different biological and clinical patterns of the disease. If confirmed at the protein level, this may provide new molecular clues for pharmaceutical targeting.

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Supplementary Information

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu).

References

1 Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 1999; **94**: 1848–1854.
2 Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H *et al*. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med* 2001; **194**: 1625–1638.
3 Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X *et al*. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. *J Exp Med* 2001; **194**: 1639–1647.
4 Vasconcelos Y, Davi F, Levy V, Oppezzo P, Magnac C, Michel A *et al*. Binet's staging system and VH genes are independent but complementary prognostic indicators in chronic lymphocytic leukemia. *J Clin Oncol* 2003; **21**: 3928–3932.
5 Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 1998; **95**: 14863–14868.
6 Haslinger C, Schweifer N, Stilgenbauer S, Döhner H, Lichter P, Kraut N *et al*. Microarray gene expression profiling of B-cell chronic lymphocytic leukemia subgroups defined by genomic aberrations and VH mutation status. *J Clin Oncol* 2004; **22**: 39–3973.
7 Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M *et al*. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med* 2003; **34**: 1764–1775.
8 Oppezzo P, Vasconcelos V, Settegrana C, Jeannel D, Vuillier F, Legarff-Tavernier M *et al*. The *LPL/ADAM29* expression ratio is a novel prognosis indicator in chronic lymphocytic leukemia. *Blood* 2005; March 31 [E-pub ahead of print].

Remission of pure red cell aplasia in T-cell receptor $\gamma\delta$-large granular lymphocyte leukemia after therapy with low-dose alemtuzumab

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TO THE EDITOR

T-cell large granular lymphocyte leukemia (T-LGL) is a lymphoproliferative disorder characterized by a persistent increase of peripheral blood large granular lymphocytes and an indolent clinical course with moderate splenomegaly, rheumatoid arthritis and neutropenia.[1] Most cases have a CD3+, TCR$\alpha\beta$+, CD4−, CD8+ phenotype, but rare variants may express CD3 and TCR$\gamma\delta$ epitopes. CD56 and CD57 are variably expressed. Rearrangements of T-cell receptor genes indicate the clonal nature of the disease. In cases requiring therapy, initial treatment consists of cyclosporin A (CSA), methotrexate (MTX) or corticosteroids.[2] Treatment indications include severe neutropenia, anemia, splenomegaly and clinical symptoms.

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Some cases of T-LGL are complicated by severe anemia due to pure red cell aplasia (PRCA).[3] PRCA is believed to result from expansion of cytotoxic lymphocytes and is also treated with immunosuppressive therapy including corticosteroids, cyclophosphamide and CSA. Recently, successful therapy of two refractory cases of PRCA with standard doses of anti-CD52 monoclonal antibody (alemtuzumab) has been reported.[4] So far, one patient with T-LGL has been reported who responded to alemtuzumab.[5] In this case, standard doses of Campath-1H were associated with considerable toxicity.[5] Hematologic side effects of alemtuzumab may sometimes require treatment withdrawal.[6] In a recent article published in this journal, Laurenti *et al*[7] reported rapid immune recovery after low-dose Campath-1H in a group of heavily pretreated patients affected by B-CLL.

Case report

Here, we report successful treatment of pure red cell aplasia with low-dose alemtuzumab in a patient with T-cell receptor $\gamma\delta$-large granular lymphocyte leukemia (TLGL$\gamma\delta$) refractory to CSA and MTX treatment. The patient was an 84-year-old Caucasian medical doctor, who was first diagnosed to have T-LGL in June 2003. Over the preceding 9 months, he had experienced a