Developing Molecular Signatures for Chronic Lymphocytic Leukemia

Edouard Cornet¹,²*, Agathe Debliquis³*, Valérie Rimelen³, Natacha Civic⁴, Mylène Docquier⁴, Xavier Troussard¹,², Bernard Drénou³, Thomas Matthes⁵,⁶*

¹ CHU Caen, Laboratory of Hematology, 14000, Caen, France, ² University of Caen, Medical School, 14000, Caen, France, ³ Département d’Hématologie, Hôpital de Mulhouse, 68051, Mulhouse, France, ⁴ Genomics Platform iGE3, University Medical Center, 1211, Geneva, Switzerland, ⁵ Hematology Service, University Hospital Geneva, 1211, Geneva, Switzerland, ⁶ Clinical Pathology Service, University Hospital Geneva, 1211, Geneva, Switzerland

☯ These authors contributed equally to this work.

* thomas.matthes@hcuge.ch

Abstract

Chronic lymphocytic leukemia (CLL) is a clonal malignancy of mature B cells that displays a great clinical heterogeneity, with many patients having an indolent disease that will not require intervention for many years, while others present an aggressive and symptomatic leukemia requiring immediate treatment. Although there is no cure for CLL, the disease is treatable and current standard chemotherapy regimens have been shown to prolong survival. Recent advances in our understanding of the biology of CLL have led to the identification of numerous cellular and molecular markers with potential diagnostic, prognostic and therapeutic significance. We have used the recently developed digital multiplexed gene-expression technique (DMGE) to analyze a cohort of 30 CLL patients for the presence of specific genes with known diagnostic and prognostic potential. Starting from a set of 290 genes we were able to develop a molecular signature, based on the analysis of 13 genes, which allows distinguishing CLL from normal peripheral blood and from normal B cells, and a second signature based on 24 genes, which distinguishes mutated from unmutated cases (LymphCLL Mut). A third classifier (LymphCLL Diag), based on a 44-gene signature, distinguished CLL cases from a series of other B-cell chronic lymphoproliferative disorders (n = 51). While the methodology presented here has the potential to provide a "ready to use" classification tool in routine diagnostics and clinical trials, application to larger sample numbers is still needed and should provide further insights about its robustness and utility in clinical practice.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world. Diagnosis is based on the results of flow cytometric analysis of malignant B cells obtained from peripheral blood, bone marrow, lymph nodes, and other organs. The characteristic phenotype
is defined by the combination of several surface markers (CD5, CD19, CD20, and CD23), and the Royal Marsden Hospital (RMH) score is widely used to distinguish CLL from other B-cell chronic lymphoproliferative disorders (B-CLPD) [1]. The clinical course is highly heterogeneous, with some patients dying from their disease within months, while others have a normal life expectancy. Predicting the disease outcome is therefore very helpful in patient management and therapeutic decision-making. Over the past decade, several prognostic markers based on genetic, phenotypic, or molecular characteristics of CLL B cells have thus been added to the original staging systems of Rai and Binet (reviewed by Chiorazzi; 2012) [2]. One of the best-studied markers is the immunoglobulin variable region heavy chain (IgVH) mutation status. In fact, roughly one half of CLL cases exhibit somatically mutated variable heavy chain genes and their presence correlates with a less aggressive clinical course [3],[4]. Specific cytogenetic changes have also been associated with unfavorable outcome (e.g.: deletion 17p) or, alternatively, with improved survival (e.g.: isolated deletion 13q) [5].

At diagnosis, the determination of the correct type of B cell disease associated with a precise outcome prediction currently depends on the interpretation of flow cytometry, cytogenetic and molecular analyses by the corresponding experts, i.e. hematologists, cytogeneticists and pathologists. These methods are costly, and labor- and time-intensive. Cheaper, objective and rapid techniques are therefore warranted. Recently, the company NanoString has developed a new high-throughput RNA expression profiling system (nCounter), which allows the direct digital readout of hundreds of mRNA molecules and their relative abundance using small amounts of total RNA (100 ng), without requiring cDNA synthesis or enzymatic reactions (DMGE; digital multiplexed gene expression) [6]. Several groups have shown high correlation with standard Affymetrix-type profiling and with quantitative RT-PCR, and have also applied this technology to mRNA extracted from Formalin-Fixed Paraffin-Embedded (FFPE) material [7],[8]. DMGE was also applied successfully to the classification of GC and ABC subtypes in diffuse large B cell lymphoma [8]. Our group has observed a high correlation between mRNA measured by DMGE and protein levels in a cohort of acute leukemia patients [9].

Using this technique for the study of a cohort of CLL samples we were able to develop a molecular signature for the diagnosis of CLL (LymphCLL Diag) as well as for the distinction between unmutated and mutated cases (LymphCLL Mut). This analysis is technically simple, can be run in every small hospital, and should be ideal for the use in clinical trials as well as for normal routine diagnosis.

Material and Methods

Patients and patient characteristics

Fresh peripheral blood (PB) samples were obtained from 30 patients of the Hematology Services of the Geneva, Mulhouse and Caen hospitals, The Ethics Committee of the Hospital of Geneva as well as of the Hospitals of Caen and Mulhouse have approved this research. Written informed consent was obtained from all patients. All patient data was analyzed anonymously. From each sample white blood cells were either (a) lysed directly in RNA lysis buffer (Qiagen, Venlo, Netherlands) and stored at -80°C, or (b) resuspended in DMSO, stored in liquid nitrogen, thawed for the present study, and then put into RNA lysis buffer (Mulhouse), or (c) lysed in RNA lysis buffer, followed by RNA extraction and storage at -80°C (Caen). For each patient a hematologic work-up was performed and the diagnosis of CLL established according to standard diagnostic guidelines ([10]). Flow cytometric analysis on CLL B cells was done for CD5, CD19, CD20, CD23, CD43, CD200, as well as for CD38 and ZAP70 expression, karyotype analysis for the detection of del17p, del13q, trisomy 12.
The analysis of the IGHV-D-J mutation status was performed on genomic DNA after isolation of leukemic cells on a Ficoll gradient. PCR amplification of IgH rearrangements was performed with either family-specific VH leader primers [11] or FR1 primers, using the BIOMED-2 protocol [12]. PCR amplicons were subjected to direct sequencing on both strands. Sequence data were analyzed using the IMGT database and the IMGT/V-QUEST tool (http://www.imgt.org). Only productive rearrangements were evaluated. VH sequences with a germ-line homology of 98% or higher were considered as unmutated, and those with a homology less than 98% were considered as mutated [13].

All the relevant patient information is presented in S1 Table.

Normal blood samples were obtained from blood donors of the Geneva blood transfusion center. Pure CD19+ B cells were prepared from four Ficoll-enriched normal blood samples using a Selection Kit from Stemcell Technologies, according to manufacturer’s instructions. Purity of the isolated cell populations was verified by flow cytometry with specific anti-CD19 and anti-CD20 antibodies and was >95% in all cases (data not shown).

Additional samples for this study were obtained from the three centers from a series of 51 patients with the following diagnoses: 20 mantle cell lymphoma (MCL), 22 marginal zone lymphoma (MZL) including 8 splenic marginal zone lymphoma (SMZL) with villous lymphocytes, 4 follicular lymphoma (FL), 5 hairy cell leukemia (HCL). RNA from these samples was processed in the same way as from the CLL samples described above.

mRNA Analysis

For the analysis with the nCounter system either 250 ng of extracted mRNA or mRNA in lysis buffer, corresponding to the equivalent of 10⁵ cells, was used, according to the manufacturer’s protocol (Nanostring H Technologies, Seattle, WA, USA). In brief, 4 μl of cell lysate or extracted mRNA was hybridized with the Nanostring CodeSet overnight at 65°C. Probes for the analysis of 290 different antigens were synthesized by NanoString technologies, including probes for nine normalization genes (S2 Table). After probe hybridizations and NanoString nCounter digital reading, counts for each mRNA species were extracted, analyzed using a homemade Excel macro, and then expressed as counts (molecules of mRNA/sample), as described previously [14]. The nCounter CodeSet contained two types of built-in controls: positive controls (spiked mRNA at various concentrations to assess the overall assay performance), and negative controls (alien probes for background calculation). Data handling and analysis was performed as described: background correction consisted of the subtraction of the negative control average plus two SD from the original counts. To select adequate normalization genes from the series of nine candidates included in the CodeSet (ACTB, TBP, RPL19, RPLP0, G6PD, ABCF1, B2M, TPT1, RPS23), their relative stability was evaluated using geNorm-method [15]. For the final normalization of the sample values the geometric mean of the counts obtained for the three selected normalization genes (RPL19, RPLP0 and TPT1) was calculated and used as normalization factor.

The technical specificities of the NanoString technology (linearity, reproducibility, sensitivity, etc.) have all been previously described [6,14,9].

The data have been submitted to GEO and can be accessed via Access Number GSE66425).

Establishing a gene list for mRNA analysis

We performed an extensive literature search and extracted a set of 290 genes from published articles and public databases, satisfying one of the following criteria: reported to be overexpressed in normal B cells compared to other blood cells; to be over- or under-expressed in CLL samples compared to normal blood samples; to be over- or under-expressed in CLL samples
compared to other B-CLPD (S2 Table). In total, a set of 299 genes (290 genes + 9 normalization genes) was used to study the mRNA profile in 5 normal peripheral blood (PB) samples, 4 purified B cell samples, and 30 samples from patients with CLL.

**Statistical analysis**

Microsoft Excel, GraphPad Prism and Partek Genomics Suite software packages were used for statistical calculations and data presentation; p-value < 0.05. An arbitrary cut-off was chosen to describe a gene as being over- or under-expressed in comparisons between two patient cohorts: ≥ 50 counts by DMGE, a ≥ 2-fold change in expression (mean of population 1 divided by mean of population 2 ≥ 2 or ≤ 2), with a p-value ≤ 0.05, using the student t-test.

**Results**

Numerous gene expression profiling studies have been performed during the last two decades on B-CLPD, and CLL in particular, based on microarray technologies. We set out to test a recently developed DMGE method for its potential utility in CLL diagnostics and prognosis determination.

**Genes expressed preferentially by normal B-cells**

Pure B cell samples were compared to samples from normal PB. In order for a gene to be considered by normal B cells to be preferentially expressed compared to other blood cells, samples with purified B cells were prepared as described above and compared to samples from normal PB. Out of 290 genes 99 genes fulfilled the criteria of an expression level ≥ 50 counts, a ≥ 2-fold change in expression (= mean of the four pure B cell samples divided by the mean of the five normal PB samples ≥ 2), with a p-value ≤ 0.05. As expected, this list contained genes coding for B-cell specific surface antigens, namely CD19, CD20 and CD79, as well as for B-cell specific transcription factors, like PAX5 and SOX11, or for immunoglobulin heavy and light chains (Table 1, for the complete list of the 99 genes see S3 Table).

Fold changes with values of 20 – 40 corresponded to the ratio of B cells present in the pure B cell and peripheral blood samples (> 95% versus 2 – 3%, respectively) and were found typically for B cell specific genes, like CD19, CD20 and CD22. Fold changes with values < 20 correspond to genes which are expressed not only by B cells, but also by other peripheral blood cells, or to genes, for which the hybridization kinetics were not optimal.

**Genes expressed preferentially in CLL samples compared to normal blood cells**

A panel of 30 CLLs, with a mean of 80% malignant B cells/sample (range: 58 – 100% per sample) and characterized for their typical cell surface phenotype, and the presence or absence of IgVH mutations, was used for this study. In order to find among the 290 gene list genes that could differentiate most effectively between CLL and normal samples, we compared the mean of all gene counts from the 30 CLL samples to the mean from the normal PB samples, and to the mean of the pure B cell samples. As previously, we selected only genes that fulfilled the arbitrary criteria of an expression level ≥ 50 counts, a ≥ 2-fold change in expression with a p-value ≤ 0.05. Fig 1A shows the Venn diagram for the genes expressed preferentially in the three different sample groups.

We obtained a list of 111 genes expressed preferentially in CLL samples compared to normal PB, and of 86 genes compared to pure B cells (S4 Table). 44 genes were common to both lists (Table 2A). Interestingly, this set of 44 genes contained genes well known to be...
overexpressed in CLL, like CD5, LPL and ROR1, but also kappa, lambda and IgG genes, showing that on a per-cell-basis CLL B cells produce more IgG mRNA than normal B cells.

As expected, principal component analysis (PCA) performed on all the 290 genes resulted in a clear separation of the samples according to their origin (pure B cells, normal PB or CLL; Fig 1B); restricting the PCA analysis to the 44 genes defined above resulted in a slightly different distribution, with normal PB and pure B cells clustered together, but with all CLL samples

| Genes                  | Mean normal PB | Mean pure B cells | Ratio pure B/normal PB | p-value |
|------------------------|----------------|-------------------|------------------------|---------|
| Surface Markers        |                |                   |                        |         |
| CD268/BAFF-R           | 940.25         | 39402.54          | 41.9                   | 0.001   |
| CD83                   | 3282.52        | 136406.91         | 41.6                   | 0.033   |
| CD79A                  | 3447.71        | 119505.02         | 34.7                   | 0.003   |
| CD69                   | 5409.08        | 181679.96         | 33.6                   | 0.010   |
| CD23/FCER2             | 226.31         | 7362.71           | 32.5                   | 0.000   |
| CD20                   | 2112.32        | 66914.33          | 31.7                   | 0.000   |
| CD22                   | 824.90         | 23927.76          | 29.0                   | 0.002   |
| CD19                   | 607.52         | 17177.12          | 28.3                   | 0.003   |
| CD40/TNFRSF5           | 541.65         | 12031.25          | 22.2                   | 0.000   |
| CD200                  | 142.54         | 3101.54           | 21.8                   | 0.002   |
| CD79B                  | 181.90         | 3672.57           | 20.2                   | 0.039   |
| CD267/TACI             | 167.24         | 2259.35           | 13.5                   | 0.004   |
| CD180                  | 443.95         | 3135.11           | 7.1                    | 0.000   |
| CD70/CD27 ligand       | 67.95          | 476.84            | 7.0                    | 0.007   |
| CD32/FCGR2B            | 1652.00        | 6086.44           | 3.7                    | 0.023   |
| CD81                   | 4430.14        | 16162.38          | 3.6                    | 0.007   |
| CD124/IL4R             | 4546.82        | 14900.94          | 3.3                    | 0.000   |
| CD24                   | 919.00         | 2537.83           | 2.8                    | 0.013   |
| CD150                  | 288.06         | 788.85            | 2.7                    | 0.009   |
| CD71/TFRC              | 2572.30        | 6537.91           | 2.5                    | 0.030   |
| CD74                   | 51335.01       | 121141.05         | 2.4                    | 0.000   |
| CD38                   | 496.21         | 1108.49           | 2.2                    | 0.048   |
| Transcription Factors  |                |                   |                        |         |
| SOX11                  | 1.48           | 128.09            | 86.7                   | 0.011   |
| PAX5                   | 267.77         | 10912.48          | 40.8                   | 0.000   |
| EBF1                   | 9.86           | 368.40            | 37.4                   | 0.021   |
| IRF4                   | 1106.86        | 7649.87           | 6.9                    | 0.024   |
| JUN                    | 6269.40        | 28921.65          | 4.6                    | 0.000   |
| BCL2                   | 1728.64        | 7690.48           | 4.4                    | 0.003   |
| MYC                    | 1640.93        | 5322.69           | 3.2                    | 0.000   |
| Immunglobulin genes    |                |                   |                        |         |
| IGHD                   | 1071.88        | 23185.99          | 21.6                   | 0.000   |
| IGHM                   | 10313.33       | 198106.21         | 19.2                   | 0.003   |
| kappa                  | 11354.34       | 70720.31          | 6.2                    | 0.000   |
| lambda                 | 17487.73       | 64435.64          | 3.7                    | 0.002   |

Listed are genes with expression values $> 50$, a pure B cell/normal PB ratio $> 2$, with a p-value $< 0.05$.  

doi:10.1371/journal.pone.0128990.t001
Fig 1. Analysis of the expression of 290 genes in normal PB, pure B cell and CLL samples. (A) Venn diagram of genes expressed preferentially in the different sample groups (normal PB, n = 5; pure B cells, n = 4; and CLL samples, n = 30). Genes were considered preferentially expressed by one sample.
clearly separated from them (Fig 1C). A detailed analysis of the expression levels of these 44 genes showed a wide range of coefficients of variation (Table 2A). 13/44 genes were expressed at a highly similar expression level in all CLL samples, with a CV < 0.5 (BMI1, CD200, CD27, CD5, COL9A2, DNMBP, FAIM3, GNRH1, LEF1, RASGRF1, ROR1, SFMBP1, TTN) (S1 Fig). These thirteen genes with the lowest CV can therefore be used as a classifier, which allows unambiguously to separate CLL samples from normal PB samples, being restricted to genes preferentially and homogenously expressed in CLL B cells compared to normal B cells (Fig 1D).

Several genes were also found to be specifically underexpressed in all CLL samples as compared to samples with pure B cells (Table 2B). These genes correspond to genes downregulated in CLL B cells compared to normal B cells, like IL-6, TIMP4 and MMP12, whose mRNA is absent in CLL B cells although normal B cells produce them in high amounts (mean: 1758.9; 92.7; 90.5 copies/sample, respectively).

Correlation between protein expression and mRNA expression
Flow cytometry allows the analysis of surface and intracytoplasmatic antigens of CLL B-cells. We studied the correlation between some antigens measured by routine flow cytometry (i.e.: CD19, CD20, CD5, CD23, CD38, CD200, kappa, lambda, ZAP70) and mRNA counts obtained by the nCounter measurements (Table 3). These expression levels corresponded to the protein expression levels detected by flow cytometry. In all the samples the CLL B cells strongly expressed CD5, CD23, CD43 and CD200 on their surface, whereas CD20 expression was found decreased compared to normal B cells.

Correlating the CD38 mRNA counts with the results from flow cytometry (S1 Table) resulted in a correlation coefficient of 0.53, similar to values of correlation coefficients found in a previous study on surface antigens in acute myeloid leukemia (AML) blasts [9] (Fig 2A). We then compared the mRNA counts in the cohort of CD38pos CLL patients with the CD38neg cohort, and the ZAP70pos cohort to the ZAP70neg one: the expected results were found, with CD38pos and ZAP70pos B-cell samples exhibiting higher mRNA counts then the corresponding negative samples (Fig 2B). Interestingly, the comparison with normal B cells gave different results for CD38 and ZAP70: normal B cells, although expressing low surface CD38 protein, showed higher CD38mRNA counts than the CD38neg CLL samples, but lower ZAP70 mRNA counts than the ZAP70neg CLL samples (Fig 2B).

Analysis of the kappa/lambda ratio at the mRNA and protein level showed a 100% correlation between flow cytometry results and mRNA counts, and all CLL cases, which were monoclonal by cytometry also showed abnormal ratios in their mRNA counts (Fig 2C).

Analysis of mutated and unmutated cases of CLL
One of the most significant prognostic factors identified in CLL that ultimately ties to the biology of disease is the mutational status of the variable region of the immunoglobulin heavy chains. We determined the mutation status in our cohort of patients and found 11 patients with mutated and 17 patients with unmutated IgVH, 2 patients were borderline (S1 Table). In order to determine which genes were correlated with the mutation status, we compared
### Table 2. List of genes over- or under-expressed specifically by CLL B cells.

| Genes | Mean | SD   | CV   | Genes | Fold change | p-value |
|-------|------|------|------|-------|-------------|---------|
| ABCA6 | 3783.3 | 1942.9 | 0.51 | BAF-FR/ CD268 | 0.426 | 0.0018 |
| ADAM29 | 482.5 | 857.1 | 1.78 | BIRC3 | 0.484 | 0.0070 |
| AICDA | 69.9 | 143.7 | 2.06 | CD22 | 0.359 | 0.0036 |
| BIK | 414.0 | 426.0 | 1.03 | CD20 | 0.124 | 0.0105 |
| BM1 | 607.6 | 202.4 | 0.33 | CD40/ TNFRSF5 | 0.481 | 0.0000 |
| BUB1B | 152.4 | 118.6 | 0.78 | CD83 | 0.086 | 0.0388 |
| CD200 | 10131.9 | 4443.1 | 0.44 | CD1 | 0.097 | 0.0191 |
| CD24 | 9424.4 | 7446.1 | 0.79 | CHL1_v4 | 0.144 | 0.0000 |
| CD269/BCMA | 1857.3 | 1201.7 | 0.65 | CHL1_v5 | 0.144 | 0.0000 |
| CD27/TNFRSF7 | 8532.6 | 3512.4 | 0.41 | CHL1_v6 | 0.144 | 0.0000 |
| CD5 | 4039.1 | 1799.7 | 0.45 | CXCR4/ SDF-1R | 0.318 | 0.0116 |
| CH1T1 | 243.2 | 162.8 | 0.67 | EBF1 | 0.031 | 0.0210 |
| CLLU1 | 5605.3 | 12595.8 | 2.25 | EB3 | 0.172 | 0.0019 |
| CNR1/ CB1 | 357.5 | 533.3 | 1.49 | EBF1/L2 | 0.133 | 0.0011 |
| COL8A2 | 4955.2 | 2398.9 | 0.48 | SERPINA9 | 0.033 | 0.0408 |
| CTLA4 | 1257.8 | 1128.3 | 0.90 | IL6 | 0.001 | 0.0032 |
| CXCR3 | 1609.2 | 849.2 | 0.53 | ITGAA/ CD49d | 0.410 | 0.0000 |
| DMD | 3625.7 | 3727.6 | 1.03 | JAM3/ JAM-C | 0.129 | 0.0001 |
| DNMBP | 4593.7 | 2102.5 | 0.46 | JUN | 0.411 | 0.0002 |
| FAIM3/ Tos1 | 44146.0 | 20379.2 | 0.46 | LGMN | 0.114 | 0.0249 |
| FCL22/ CD23 | 15988.5 | 8133.0 | 0.51 | MMP12 | 0.019 | 0.0003 |
| FGFR2 | 707.3 | 693.6 | 0.98 | MS4A1/ CD20 | 0.293 | 0.0001 |
| FGFR1 | 554.3 | 784.8 | 1.42 | MYC | 0.198 | 0.0001 |
| FIP111 | 2284.9 | 1636.2 | 0.72 | NRE7 | 0.280 | 0.0500 |
| FLT3 | 406.5 | 512.4 | 1.26 | RAGE/ MOK | 0.451 | 0.0086 |
| FMO3 | 1740.1 | 12667.9 | 0.73 | REL | 0.335 | 0.0005 |
| GNRH1 | 518.1 | 232.4 | 0.45 | SOX11 | 0.189 | 0.0105 |
| IGFBP4 | 4858.1 | 3366.4 | 0.69 | TIP4 | 0.011 | 0.0107 |
| IGH1 to 4 | 13373.7 | 11186.2 | 0.84 | ZFP36 | 0.426 | 0.0056 |
| IGSF3 | 1129.0 | 692.7 | 0.61 | kappa | 12893.8 | 120715.3 | 0.94 |
| IL2RA/ IFL2R | 2205.9 | 1462.3 | 0.66 | lambda | 142654.3 | 170967.7 | 1.20 |
| LERF1 | 7270.0 | 2323.7 | 0.32 | LERF1 | 7270.0 | 2323.7 | 0.32 |
| LPL | 641.1 | 681.2 | 1.06 | RAPGEF3 | 817.9 | 457.3 | 0.56 |
| RASGFRF1 | 2960.0 | 14367.9 | 0.49 | ROR1 | 2376.8 | 1107.6 | 0.47 |
| Selectin P/ CD62 | 1067.6 | 708.9 | 0.66 | SEPT 10 | 667.5 | 1305.7 | 1.96 |
| SFMBT1 | 11065.5 | 3547.0 | 0.32 | TTN | 7243.9 | 3010.2 | 0.42 |
| WNT3 | 5240.6 | 5963.0 | 1.14 | WNT3 | 5240.6 | 5963.0 | 1.14 |

(A) List of 44 genes expressed specifically by CLL B cells. Expression of mRNA was compared between the mean of 30 CLL samples and the mean of 5 normal PB and 4 pure B cell samples. Listed are those genes that fulfill the following criteria: > 2-fold change in expression between CLL samples and normal PB samples and pure B cell samples; p-value < 0.05; expression level in CLL samples > 50. (B) List of genes underexpressed by CLL samples compared to pure B cell samples. Listed are those genes that fulfill the following criteria: < 0.5-fold change in expression between CLL samples and pure B-cell samples; p-value < 0.05; expression level in pure B cell samples > 50.

doi:10.1371/journal.pone.0128990.t002
mutated to unmutated samples and listed genes either overexpressed in mutated versus unmutated, or in unmutated compared to mutated samples (Table 4). 24 genes were found to be differentially expressed: nineteen genes were overexpressed in unmutated, and five in mutated samples. Among the differentially expressed genes were nine genes from the 44-gene-list, which we used to distinguish CLL from normal samples, as well as several genes described in the literature: CD38, ZAP-70, LPL, etc. ([4],[16],[17],[18]; see also Table 4).

This 24-gene panel called “LymphCLL Mut” allowed a clear distinction between both types of CLL (Fig 3; S2 Fig).

Total light chain production was increased in mutated vs unmutated samples (185207 counts vs 324812 counts; p = 0.008).

Analysis of LDOC1 expression

LDOC1 mRNA has been reported to be highly expressed in aggressive cases of CLL and to correlate with IgVH mutation status and with prognosis [22]. When we analyzed the mRNA expression in our 30 CLL cases, we found indeed a dichotomic distribution, completely different from the homogenous distribution, which we described in the thirteen genes used for the CLL classifier (S1 Fig). Interestingly, when we looked among all the 290 genes analyzed, only eight genes were found to correlate with LDOC1 expression: six genes with a positive correlation (SEPT10, LPL, CD26, EPB41L2, CXCR6, CRY1) and two genes with a negative correlation (ADAM29, CD150; Table 5; S3 Fig). ADAM29 was exclusively expressed in samples with absent/low LDOC1 expression and vice versa (Fig 4A). Superficially, this expression pattern corresponded to the IgVH mutation status of these samples, but a closer inspection yielded a group of five samples with absence of both LDOC1 and ADAM29 mRNAs (two mutated and three unmutated cases). The LDOC1/ADAM29 ratio clearly reflects this separation into three different groups of samples. Interestingly, in a previous report Oppezzo et al have published

| Genes        | Mean normal PB | Mean pure B cells | Mean CLL | Ratio CLL/normal PB | Ratio CLL/pure B cells | p-value |
|--------------|----------------|-------------------|---------|---------------------|------------------------|---------|
| CD43         | 1886.17        | 17.20             | 1253.7  | 0.7                 | 72.9                   | 0.000   |
| CD5          | 1782.16        | 250.99            | 4039.1  | 2.3                 | 16.1                   | 0.000   |
| CD200        | 142.54         | 3101.54           | 10131.9 | 71.1                | 3.3                    | 0.000   |
| FCER2/CD23   | 226.31         | 7362.71           | 15988.5 | 70.6                | 2.2                    | 0.000   |
| CD79B        | 181.90         | 3672.57           | 4665.0  | 25.6                | 1.3                    | 0.421   |
| CD19         | 607.52         | 17177.12          | 19164.6 | 31.5                | 1.1                    | 0.480   |
| CD38         | 496.21         | 1108.49           | 860.8   | 1.7                 | 0.8                    | 0.760   |
| CD79A        | 3447.71        | 119505.02         | 64178.8 | 18.6                | 0.5                    | 0.016   |
| CD22         | 824.90         | 23927.76          | 8597.4  | 10.4                | 0.4                    | 0.004   |
| MS4A1/CD20   | 2112.32        | 66914.33          | 19628.9 | 9.3                 | 0.3                    | 0.000   |

doi:10.1371/journal.pone.0128990.t003
the LPL/ADAM29 ratio as a surrogate marker for IgVH status [24]. Comparing this ratio in our samples to the IgVH status showed concordance in 27/30 samples; the three discordant samples corresponded to samples with absent LDOC1 or ADAM29 expression (Fig 4B).

Fig 2. Correlation between mRNA and protein expression. (A) Correlation between CD38 protein expression, as measured by flow cytometry (% positive CLL B cells) and CD38 mRNA counts, as measured by the nCounter (arbitrary units). (B) Quantification of CD38 and ZAP70 mRNA counts in normal peripheral blood (PB), pure B cells, and in CLL B cells. CLL B cells were analyzed for ZAP70 and for CD38 expression by flow cytometry and then grouped for the mRNA determination, according to presence or absence of these two antigens. (C) Immunoglobulin light chain ratios in 30 CLL patient samples (rhombi) and in normal B cells (triangles: normal PB samples; circles: pure B cell samples). The mean +/- 2SD interval for ratios from polyclonal normal B cells and normal PB is shown (mean 0.89; SD = 0.22; small dots).
Validating the CLL classifier

In order to develop a clinically useful classifier, CLL samples not only have to be distinguished unambiguously from normal PB samples, but also from other lymphoma subtypes. We therefore tested the 44 genes found to distinguish CLL from normal PB samples on a series of 51 patients with different B-CLPD, i.e., MCL, MZL, FL and HCL. The PCA analysis showed a clear separation of the CLL from all the other B-CLPD samples, with the exception of one confirmed CLL case, which was misdiagnosed (Fig 5). None of the B-CLPD samples was misdiagnosed as a CLL.

Discussion

In the present work we describe the use of DMGE, a recently developed technique for the quantitative and parallel analysis of hundreds of mRNAs, in a cohort of 30 CLL patients. Starting
from a set of 290 genes with preferential expression in B cells and CLL cells described in previously published reports, we were able to establish lists of genes, with preferential expression in normal and in CLL B cells, respectively, and which allowed distinguishing unambiguously CLL samples from normal PB samples and CLL B cells from normal B cells. Restricting these gene lists to genes expressed homogenously by all CLL samples, independent of chromosomal abnormalities, yielded a classifier, based on the analysis of only 13 genes. Applying this classifier in an unsupervised analysis of our cohort resulted in a perfect separation of all 30 CLL samples. Adding kappa/lambda ratios to the classifier will certainly increase its discriminative power, since our results show for all cases a clear distinction between polyclonal samples (normal PB and sorted normal B cells) and CLL samples with essentially monoclonal B cell populations.
In a previous study, we have already used DMGE in acute myeloid leukemia (AML) to correlate leukemic blast mRNA expression with surface antigens determined by flow cytometry [9]. The present study confirms close correlation between flow cytometry results and DMGE analysis for some surface proteins.

The DMGE technique also allows the study of genes with prognostic relevance in parallel with the 13 gene diagnostic classifier, using a single assay. Interestingly, these genes fall broadly into two categories: those expressed with wide variations in different samples (up to $10^4$ difference in mRNA expression; e.g.: LILR4 and CLLU1), and those with a present/absent, dichotomic pattern (e.g.: LDOC1, LPL, ADAM29).

Analysis of the IgVH mutation status is widely used to distinguish patients with a good from those with a bad prognosis. Several surrogate markers have been described in the literature and shown to correlate with the IgVH mutation status. We could confirm most of them, such as ZAP70, CD38, LPL and LDOC1 (Table 4). On the contrary, we did not find any differential expression for the following genes FCRL2 ($p = 0.08$) and HS1 ($p = 0.10$), also reported to vary between mutated and unmutated CLL samples [36], [37]. In an unsupervised analysis 28/30 (93%) CLL samples were correctly classified using the "Lymph CLL Mut" classifier based on 24 genes with a differential expression between IgVH mutated and unmutated cases.

The LPL/ADAM29 ratio has already been described previously to constitute a surrogate marker for the IgVH mutation status [24], and also to be related to prognosis [38]. Whereas this ratio distinguishes two different types of CLL samples, the determination of the LDOC1/ADAM29 ratio allowed distinction of 3 subclasses: IgVH mutated with high expression of ADAM29, unmutated samples with high expression of LDOC1, and a third category (mixed mutated and unmutated samples) without expression of LDOC1 and ADAM29. This third group did not show any common IgVH usage or chromosomal abnormalities. Future studies have to tell us whether there is any clinical significance or any existing correlations between this category and prognosis.

In our final analysis we tested the 44-gene signature, which differentiated CLL from normal PB samples, on a set of 51 samples from patients with various common B-CLPD. Similar to the flow cytometric RMH score our "LymphCLL Diag" molecular classifier distinguished CLL.

### Table 5. LDOC1 expression in CLL samples.

| Genes   | Mean   | Mean   | Ratio   | p-value |
|---------|--------|--------|---------|---------|
|         | LDOC1  | LDOC1  | LDOC1   |         |
| neg     |        | pos    | pos/neg |         |
| LDOC1   | 1      | 483    | 483.4   | 0.0000  |
| SEPT 10 | 68     | 1365   | 20.2    | 0.0138  |
| LPL     | 154    | 1331   | 8.6     | 0.0000  |
| CD26/ DPP4 | 45 | 162    | 3.6     | 0.0023  |
| EPB41L2 | 132    | 353    | 2.7     | 0.0280  |
| CXCR6   | 33     | 89     | 2.7     | 0.0374  |
| CRY1    | 551    | 1404   | 2.5     | 0.0001  |

CLL samples were separated into those highly expressing LDOC1 and those with absent/low expression. Listed are those genes that fulfill the following criteria: >2-fold change in expression between LDOC1 pos samples compared to LDOC1 neg samples, with a p-value < 0.05 or >2-fold change in expression between LDOC1 neg samples compared to LDOC1 pos samples, with a p-value < 0.05.

In a previous study, we have already used DMGE in acute myeloid leukemia (AML) to correlate leukemic blast mRNA expression with surface antigens determined by flow cytometry [9]. The present study confirms close correlation between flow cytometry results and DMGE analysis for some surface proteins.

The DMGE technique also allows the study of genes with prognostic relevance in parallel with the 13 gene diagnostic classifier, using a single assay. Interestingly, these genes fall broadly into two categories: those expressed with wide variations in different samples (up to $10^4$ difference in mRNA expression; e.g.: LILR4 and CLLU1), and those with a present/absent, dichotomic pattern (e.g.: LDOC1, LPL, ADAM29).

Analysis of the IgVH mutation status is widely used to distinguish patients with a good from those with a bad prognosis. Several surrogate markers have been described in the literature and shown to correlate with the IgVH mutation status. We could confirm most of them, such as ZAP70, CD38, LPL and LDOC1 (Table 4). On the contrary, we did not find any differential expression for the following genes FCRL2 ($p = 0.08$) and HS1 ($p = 0.10$), also reported to vary between mutated and unmutated CLL samples [36], [37]. In an unsupervised analysis 28/30 (93%) CLL samples were correctly classified using the "Lymph CLL Mut" classifier based on 24 genes with a differential expression between IgVH mutated and unmutated cases.

The LPL/ADAM29 ratio has already been described previously to constitute a surrogate marker for the IgVH mutation status [24], and also to be related to prognosis [38]. Whereas this ratio distinguishes two different types of CLL samples, the determination of the LDOC1/ADAM29 ratio allowed distinction of 3 subclasses: IgVH mutated with high expression of ADAM29, unmutated samples with high expression of LDOC1, and a third category (mixed mutated and unmutated samples) without expression of LDOC1 and ADAM29. This third group did not show any common IgVH usage or chromosomal abnormalities. Future studies have to tell us whether there is any clinical significance or any existing correlations between this category and prognosis.

In our final analysis we tested the 44-gene signature, which differentiated CLL from normal PB samples, on a set of 51 samples from patients with various common B-CLPD. Similar to the flow cytometric RMH score our "LymphCLL Diag" molecular classifier distinguished CLL.
Fig 4. Analysis of LDOC1 expression. (A) LDOC1 mRNA expression was measured in 30 CLL samples and samples ordered according to absent/low (No 1–17) or high expression (No 18–30); black columns. ADAM29 mRNA expression shows an inverse pattern (No 1–12); hashed columns. Mutation status is noted below (BL = borderline; M = mutated; UM = unmutated IgVH) (B) Analysis of LDOC1/ADAM29 and LPL/ADAM29 ratios.

doi:10.1371/journal.pone.0128990.g004
from other B-CLPD with high sensitivity and specificity (97% and 100%, respectively). Associating the “LymphCLL Diag” gene panel with the “LymphCLL Mut” panel, the kappa/lambda ratio and the LDOC1/ADAM29 ratio, a complete diagnostic and prognostic procedure could be performed in one single “ready to use” assay, based on a panel of 61 genes.

Several of the laboratory analyses described for diagnostic and prognostic purposes in CLL are time- and labor-intensive and not well suited for routine testing in most clinical laboratories. One example is the determination of the IgVH mutational status, which is rather expensive, needs specialized know-how, and is currently only performed in a restricted number of laboratories under the expertise of molecular biologists [39]. Threshold levels are arbitrary (in most reports > 2% are considered mutated) and a grey zone exists [39].

Another example is the ZAP70 expression analysis by flow cytometry, which has been largely abandoned due to difficulties in standardization [40] [41] or the ZAP expression analysis by
RT-PCR, which requires purification of B cells prior to the assay [39], rendering this approach unsuitable for routine diagnostics.

The new sequencing technologies also hold the promise to give valuable data for prognosis determination of CLL patients, most notably TP53, NOTCH1, ATM, SF3B1 and BIRC3 mutations [42][43]. Mutations in these genes occur in approx. 2%-17% of CLL patients at diagnosis and the prognostic importance of some of them have already been studied in prospective trials [44] [45]. Whether this information is complementary to established prognostic factors and results from mRNA and gene expression studies like ours have ideally to be investigated in large future prospective and comparative trials. Although already widely used for research purposes deep sequencing techniques are not yet used in routine laboratories and the expensive, labor-intensive technology and bioinformatically complex softwares will make this transfer challenging.

With the development of DMGE a new technique has arrived, which allows genetic profiling with the parallel analysis of hundreds of mRNAs by a technically extremely simple method. DMGE has a short turn-around time of < 2 days, needs minimal hands-on-time for technicians and is much less costly than whole gene expression profiling or deep sequencing. Moreover, this technique allows for automated data-analysis, and has a read-out, which is intuitive and does not need complicated bio-informatics tools for the analysis or interpretation. By focusing our approach on the analysis of a highly selected set of genes expressed preferentially by B cells, we could obtain signatures from the analysis of whole blood samples, rendering an additional B cell purification step unnecessary.

The parallel quantitative analysis of tens to hundreds of mRNAs allows the integration of several diagnostic and prognostic factors in one assay, contrary to numerous studies from the past, which have only analyzed one or two factors at a time. It should be therefore ideally suited for large trials aiming at the comparison of multiple factors in many different patient samples and for molecular characterization of cases without available living cells for flow cytometry, such as cDNA or FFPE. Smaller labs could also profit from this approach for routine diagnostics based on an automated data analysis.

To fully appreciate the clinical usefulness and discriminative power of this approach, prospective studies with a much larger number of CLL samples will have to be performed in the future, including samples with other B-CLPD and reactive/inflammatory conditions. Additional prognostic markers can be easily incorporated to the classifier and then be studied simultaneously in clinical trials, but also in routine diagnosis. Integrating information from gene profiling studies with results from genomic mutation and NGS analyses should ultimately lead to better prognostication schemes for patients.

Supporting Information

S1 Fig. Comparison of mRNA counts from normal PB, pure B cell and CLL samples. Shown are 13 selected genes from the 44-gene list with a low CV < 0.5. (TIF)

S2 Fig. Heatmap of 30 CLL samples, analyzed with the 24-gene classifier for the recognition of mutated vs unmutated samples. (TIF)

S3 Fig. Heatmap of CLL samples based on analysis of genes correlated with expression of LDOC1 mRNA (unsupervised analysis). (TIF)
S1 Table. Patient characteristics. 30 patients with a diagnosis of typical CLL were included in the study. Flow cytometry was used to determine the % of malignant CLL B cells/sample, and the % of CD38+ CLL B-cells; values ≥20% were considered positive. Mutation status of the IgVH was determined as described in Material and Methods section. ND = not done.

(XLS)

S2 Table. Probes for Nanostring analysis. 290 genes were chosen to constitute the CodeSet for this work, plus nine housekeeping genes.

(XLSX)

S3 Table. mRNA expression of 290 genes for all 30 CLL patient samples.

(XLSX)

S4 Table. List of genes expressed preferentially by CLL samples compared to normal PB (A) and pure B cell samples (B).

(XLSX)

Acknowledgments

We thank Sylvie Ruault-Jungblut for expert technical assistance.

Author Contributions

Conceived and designed the experiments: XT BD TM. Performed the experiments: EC AD VR TM. Analyzed the data: NC MD EC AD VR XT BD TM. Contributed reagents/materials/analysis tools: EC AD XT BD NC MD TM. Wrote the paper: EC AD VR BD XT TM.

References

1. Matutes E, Owusu-Ankomah K, Morilla R, Garcia Marco J, Houlihan A, Que TH, et al. (1994) The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia 8: 1640–1645. PMID: 7523797
2. Chiorazzi N (2012) Implications of new prognostic markers in chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program 2012: 76–87. doi: 10.1182/asheducation-2012.1.76 PMID: 23233564
3. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK (1999) Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 94: 1848–1854. PMID: 10477713
4. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. (1999) Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 94: 1840–1847. PMID: 10477712
5. Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, et al. (2000) Genomic aberrations and survival in chronic lymphocytic leukemia. N Eng J Med 343: 1910–1916. PMID: 11136261
6. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. (2006) Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol 26: 317–325. doi: 10.1038/nbt1385 PMID: 18278033
7. Masque-Soler N, Szczepanowski M, Kohler CW, Spang R, Klapper W (2013) Molecular classification of mature aggressive B-cell lymphoma using digital multiplexed gene expression on formalin-fixed paraffin-embedded biopsy specimens. Blood 122: 1985–1986. doi: 10.1182/blood-2013-06-508937 PMID: 24030260
8. Scott DW, Wright GW, Williams PM, Lih CJ, Walsh W, Jaffe ES, et al. (2014) Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. Blood 123: 1214–1217. doi: 10.1182/blood-2013-11-536433 PMID: 24398326
9. Fernandez P, Solenthaler M, Sperini O, Quarrroz S, Rovero A, Lovey PY, et al. (2012) Using digital RNA counting and flow cytometry to compare mRNA with protein expression in acute leukemias. PLoS One 7: e49010. doi: 10.1371/journal.pone.0049010 PMID: 23152841
11. Fais F, Ghiotto F, Hashimoto S, Sellars B, Valetto A, Allen SL, et al. (1998) Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest 102: 1515–1525. PMID: 9788964

12. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. (2003) De-novo expression of immunoglobulin light chain genes in secondary head and neck squamous cell carcinoma. J Pathol 199: 229–236. PMID: 12551615

13. van't Veer MB, Brooijmans AM, Langerak AW, Verhaaf B, Goudswaard CS, Graveland WJ, et al. (2003) De-novo expression of immunoglobulin light chain genes in diffuse large B-cell lymphomas. Br J Haematol 121: 650–657. PMID: 12855567

14. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, et al. (2003) ZAP-70 expression and sign and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombination in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 17: 2257–2317. PMID: 14671650

15. van der Sompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034.

16. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, et al. (2003) ZAP-70 expression as a surrogate for immunoglobulin-variable-region-changes in chronic lymphocytic leukemia. N Engl J Med 348: 1764–1775. PMID: 12724482

17. Rassenti LZ, Jain S, Keating MJ, Wierda WG, Grever MR, Byrd JC, et al. (2008) Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia. Blood 112: 1923–1930. doi:10.1182/blood-2007-05-092882 PMID: 18577710

18. van't Veer MB, Brooijmans AM, Langerak AW, Verhaal F, Goudsward CS, Graveland WJ, et al. (2006) The predictive value of lipoprotein lipase for survival in chronic lymphocytic leukemia. Haematologica 91: 56–63. PMID: 16434371

19. Kienle D, Benner A, Lauflle C, Schneider C, Buhler A, et al. (2010) Gene expression factors as predictors of genetic risk and survival in chronic lymphocytic leukemia. Haematologica 95: 102–109. doi:10.3324/haematol.2009.010298 PMID: 19951976

20. Albesiano E, Messmer BT, Damle RN, Allen SL, Rai KR, Chiorazzi N (2003) Activation-induced cytidine deaminase in chronic lymphocytic leukemia B cells: expression as multiple forms in a dynamic, variably sized fraction of the clone. Blood 102: 3333–3339. PMID: 12955567

21. McCarthy H, Wierda WG, Barron LL, Cromwell CC, Wang J, Coombes KR, et al. (2003) High expression of activation-induced cytidine deaminase (AID) and splice variants is a distinctive feature of poor-prognosis chronic lymphocytic leukemia. Blood 101: 4903–4908. PMID: 12566616

22. Duzkale H, Schweighofer CD, Coombes KR, Barron LL, Ferrajoli A, O'Brien S, et al. (2011) LDOC1 mRNA is differentially expressed in chronic lymphocytic leukemia and predicts overall survival in untreated patients. Blood 117: 4076–4084. doi:10.1182/blood-2010-09-304881 PMID: 21310924

23. Haslinger C, Schweiker N, Stilgenbauer S, Dohner H, Lichter P, Kraut N, et al. (2004) Microarray gene expression profiling of B-cell chronic lymphocytic leukemia subgroups defined by genomic aberrations and VH mutation status. J Clin Oncol 22: 3937–3949. PMID: 15459216

24. Oppezzo P, Vasonconelos Y, Settegrana C, Jeannel D, Vuillier F, Legarff-Tavernier M, et al. (2005) The LPL/ADAM29 expression ratio is a novel prognosis indicator in chronic lymphocytic leukemia. Blood 106: 650–657. PMID: 15802535

25. Heintel D, Kienle D, Shehata M, Krober A, Kroemer E, Schwarzinger I, et al. (2005) High expression of lipoprotein lipase in poor risk B-cell chronic lymphocytic leukemia. Leukemia 19: 1223. PMID: 1858619

26. Popaczy E, Tauber S, Bilban M, Kostner G, Gruber M, Eder S, et al. (2013) Lipoprotein lipase in chronic lymphocytic leukemia—strong biomarker with lack of functional significance. Leuk Res 37: 631–636. doi:10.1016/j.leukres.2013.02.008 PMID: 23478142

27. Patricia Weiss EAP, Trang Le, Cathrin Skrabs, Michaela Gruber, Clemens Pausz, Ulrich Jäger and Katrina Vanura (2012) Cannabinoid Receptor 1 in Chronic Lymphocytic Leukemia: Strong Prognostic Marker with Limited Therapeutic Use. ASH Abstract Book.

28. Hanoun M, Eisele L, Suzuki M, Greally JM, Huttmann A, Aydin S, et al. (2012) Epigenetic silencing of the circadian clock gene CRY1 is associated with an indolent clinical course in chronic lymphocytic leukemia. PLoS One 7: e34347. doi:10.1371/journal.pone.0034347 PMID: 22470559
29. Eisele L, Prinz R, Klein-Hitpass L, Nuckel H, Lowinski K, Thomale J, et al. (2009) Combined PER2 and CRY1 expression predicts outcome in chronic lymphocytic leukemia. Eur J Haematol 83: 320–327. doi: 10.1111/j.1600-0609.2009.01287.x PMID: 19500131

30. Gattel V, Bulian P, Del Principe MI, Zucchett A, Maurillo L, Buccisano F, et al. (2008) Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. Blood 111: 865–873. PMID: 17959854

31. Cro L, Morabito F, Zucal N, Fabris S, Lionetti M, Cutrona G, et al. (2009) CD26 expression in mature B-cell neoplasia: its possible role as a new prognostic marker in B-CLL. Hematol Oncol 27: 140–147. doi: 10.1002/hon.888 PMID: 19247982

32. Sulda ML, Abbott CA, Macardle PJ, Hall RK, Kuss BJ (2010) Expression and prognostic assessment of dipeptidyl peptidase IV and related enzymes in B-cell chronic lymphocytic leukemia. Cancer Biol Ther 10: 180–189. PMID: 20534982

33. Cro L, Prinz R, Klein-Hitpass L, Nuckel H, Lowinski K, Thomale J, et al. (2009) Combined PER2 and CRY1 expression predicts outcome in chronic lymphocytic leukemia. Eur J Haematol 83: 320–327. doi: 10.1111/j.1600-0609.2009.01287.x PMID: 19500131

34. Schweighofer CD, Coombes KR, Barron LL, Diao L, Newman RJ, Ferrajoli A, et al. (2011) A two-gene signature, SKI and SLAMF1, predicts time-to-treatment in previously untreated patients with chronic lymphocytic leukemia. PLoS One 6: e28277. doi: 10.1371/journal.pone.0028277 PMID: 22194822

35. Jelinek DF, Tschumper RC, Stolovitzky GA, Iturria SJ, Tu Y, Lepre J, et al. (2003) Identification of a global gene expression signature of B-chronic lymphocytic leukemia. Mol Cancer Res 1: 346–361. PMID: 12651908

36. Li FJ, Ding S, Pan J, Shakhmatov MA, Kashentseva E, Wu J, et al. (2008) FCRL2 expression predicts IGHV mutation status and clinical progression in chronic lymphocytic leukemia. Blood 112: 179–187. doi: 10.1182/blood-2008-01-131359 PMID: 18314442

37. Frezza F, Gattazzo C, Martini V, Trimarco V, Teramo A, Carraro S, et al. (2012) HS1, a Lyn kinase substrate, is abnormally expressed in B-chronic lymphocytic leukemia and correlates with response to fludarabine-based regimen. PLoS One 7: e39902. doi: 10.1371/journal.pone.0039902 PMID: 22768161

38. Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE, et al. (2003) ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 101: 4944–4951. PMID: 12595313

39. Letestu R, Rawstron A, Ghia P, Villamor N, Boeckx N, Boettcher S, et al. (2006) Evaluation of ZAP-70 expression by flow cytometry in chronic lymphocytic leukemia: A multicentric international harmonization process. Cytometry B Clin Cytom 70: 309–314. PMID: 16906588

40. Ferreira PG, Jares P, Rico D, Gomez-Lopez G, Martinez-Trillos A, Villamor N, et al. (2014) Transcriptional characterization by RNA sequencing identifies a major molecular and clinical subdivision in chronic lymphocytic leukemia. Genome Res 24: 212–226. doi: 10.1101/gr.152132.112 PMID: 24265505

41. Sutton LA, Ljungstrom V, Mansouri L, Young E, Cortese D, Navrkalova V, et al. (2015) Targeted next-generation sequencing in chronic lymphocytic leukemia: a high-throughput yet tailored approach will facilitate implementation in a clinical setting. Haematologica 100: 1370–1376. doi: 10.3324/haematol.2014.109777 PMID: 25490502

42. Oscier DG, Rose-Zerilli MJ, Winkelmann N, Gonzalez de Castro D, Gomez B, Forster J, et al. (2013) The clinical significance of NOTCH1 and SF3B1 mutations in the UK LRF CLL4 trial. Blood 121: 468–475. doi: 10.1182/blood-2012-05-429282 PMID: 23086750

43. Stillgenbauer S, Schnaiter A, Paschka P, Zenz T, Rossi M, Dohner K, et al. (2014) Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. Blood 123: 3247–3254. doi: 10.1182/blood-2014-01-546150 PMID: 24652989