Identification of uniquely expressed transcription factors in highly purified B-cell lymphoma samples

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Transcription factors (TFs) are critical for B-cell differentiation, affecting gene expression both by repression and transcriptional activation. Still, this information is not used for classification of B-cell lymphomas (BCLs). Traditionally, BCLs are diagnosed based on a phenotypic resemblance to normal B-cells; assessed by immunohistochemistry or flow cytometry, by using a handful of phenotypic markers. In the last decade, diagnostic and prognostic evaluation has been facilitated by global gene expression profiling (GEP), providing a new powerful means for the classification, prediction of survival, and response to treatment of lymphomas. However, most GEP studies have typically been performed on whole tissue samples, containing varying degrees of tumor cell content, which results in uncertainties in data analysis. In this study, global GEP analyses were performed on highly purified, flow-cytometry sorted tumor-cells from eight subgroups of BCLs. This enabled identification of TFs that can be uniquely associated to the tumor cells of chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), hairy cell leukemia (HCL), and mantle cell lymphoma (MCL). The identified transcription factors influence both the global and specific gene expression of the BCLs and have possible implications for diagnosis and treatment. Am. J. Hematol. 85:418–425, 2010. © 2010 Wiley-Liss, Inc.

Introduction

B-cell lymphomas (BCLs) constitute a diverse set of tumors, both morphologically and clinically, that are mainly classified in relation to normal B-cell differentiation [1]. However, it is now clear that some lymphoid malignancies, for example, hairy cell leukemia (HCL) cannot easily be traced to a specific normal B-cell counterpart or differentiation stage, why much more detailed molecular analysis is called for [2]. Gene expression profiling has lately proven to be a powerful tool in the understanding of BCL biology, and, for instance, it has been used to demonstrate how diffuse large B-cell lymphoma (DLBCL) can be subgrouped based on unique gene expression profiles [3], which also can be associated to prognosis [4]. In both DLBCL and mantle cell lymphoma (MCL), prognostic signatures have been defined by the gene expression of the lymphoma cells [3,5]. This is in contrast to FL where the aggressiveness of the disease is mainly determined by the cellular microenvironment [6–8], composed of both immunocytes and stromal cells. Thus, for most lymphoma entities, the utilization of pure cell populations would provide an unmet need to identify specific genes related to each subtype of tumor cells and to trace their cellular origin.

Similar to immunophenotyping and gene expression profiling, it has recently been shown that patterns of microRNA expression define different stages of B-cell differentiation and also that these differences can be used to classify BCLs [9]. In contrast to most microRNAs, which mainly work through repression of genes, transcription factors (TFs) regulates gene expression both by repression and transcriptional activation of genes. Several of these genes, such as *PAX5* [10], *BCL6* [11], *IRF-4* [12], *BLIMP-1* [13], and *XBP1* [14], are critical in B-cell differentiation. However, the TF *SOX11*, which is not involved in normal B-cell differentiation, has recently been identified as a strong diagnostic marker for MCL [15] highlighting the importance of analyzing not only B-cell-associated markers in the assessment of lymphoma gene regulation.

Since TFs are important players in normal B-cell differentiation but also in disease progression [12], we focused our analysis specifically on the regulation of those, to potentially identify new functional, diagnostic, and therapeutic targets. In this study, highly purified, flow-cytometry sorted,

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Additional supporting information may be found in the online version of this article.

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TABLE I. Clinical Information of the B-Cell Lymphoma Samples

Samples	Grade[1]	Sex (F/M)	Stage[2] I–II	Stage[2] III–IV	IGVH mutational status "mutated"	IGVH mutational status "unmutated"
CLL ($n = 15$)	NA[3]	4/11	NA[3]	NA[3]	7	8
DLBCL-GC ($n = 7$)	NA[3]	5/2	2	4	7	0
DLBCL-nonGC ($n = 10$)	NA[3]	5/5	4	4	10	0
DLBCL-tr ($n = 6$)	NA[3]	2/4	0	5	6	0
FL ($n = 12$)	I–II	6/6	2	6	NA[3]	NA[3]
HCL ($n = 10$)	NA[3]	4/6	NA[3]	NA[3]	7	3
L-MCL ($n = 6$)	NA[3]	6/0	0	6	NA[3]	NA[3]
MCL ($n = 12$)	NA[3]	4/8	1	10	2	10

[1] Grade at diagnosis, when available

[2] Stage, when available

[3] NA = not available

B-cells from eight different BCL entities (follicular lymphoma (FL), transformed DLBCL (DLBCL-tr), DLBCL with a germinal center (GC) phenotype (DLBCL-GC), DLBCL with a nonGC phenotype (DLBCL-nonGC), MCL, chronic lymphocytic lymphoma (CLL), leukemic version of MCL (L-MCL), HCL, and four subpopulations derived from nonmalignant tonsils (naïve B-cells (N), centrocytes (CC), centroblasts (CB), memory B-cells (M)) were analyzed, using global gene expression analysis). By using purified tumor cells, we were able to identify unique TFs for the different diagnostic B-cell lymphoma subgroups. These TFs are not only potential new molecular targets, but partly also influence the difference in global and specific gene expression observed between B-cell lymphomas.

Material and Methods

Collection and isolation of tumor cells

The study was conducted according to the Helsinki declaration and with approval of the Uppsala University Ethics Committee (approval 01-399). All specimens in this study were diagnosed according to the World Health Organization (WHO) criteria [16] including; FL, $n = 12$; DLBCL-tr, $n = 6$; DLBCL-GC, $n = 7$; DLBCL-nonGC, $n = 10$; MCL, $n = 12$; CLL, $n = 15$; L-MCL, $n = 6$ and HCL, $n = 10$. Of note, some samples (FL, $n = 12$; DLBCL-tr, $n = 6$) have been analyzed previously in a study where the transformation from FL to DLBCL-tr was examined [17]. Freshly frozen tumor cell suspensions were collected from the biobank at the Department of Pathology, Uppsala University Hospital (Uppsala, Sweden) (FL, DLBCL, MCL, CLL, HCL), and from the biobank at Rigshospitalet (Copenhagen, Denmark) (L-MCL). The subgrouping of DLBCL into GC and nonGC subgroups was based on immunohistochemistry for CD10, BCL6, and IRF-4 according to previous publications [18,19]. After diagnosis, the cells were sorted, using a FACSAria (Becton Dickinson, Franklin Lakes, New Jersey), based on their expression of CD19, kappa (κ) light chain or lambda (λ) light chain and lack of CD3, using antibodies toward these antigens, CD19-PB (Dako, Glostrup, Denmark), κ -APC (Becton Dickinson), λ -PE (Dako), and CD3-FITC (Becton Dickinson). This enabled the identification of a BCL population based on identical light-chain clonality. The fraction of tumor cells in the samples before sorting varied (Results section) but all samples were generally purified to >97% as assessed by FACS analysis. The isolated cells were then lysed in Trizol (Invitrogen Corporation, CA), as previously described [20]. The patient material details are shown in Table I.

Collection and isolation of nonmalignant tonsil B-cells

The nonmalignant B-cell populations (N, CC, CB, M) were derived from three fresh pediatric tonsils specimens obtained from Lund University Hospital (Lund, Sweden) and purified as previously described [20]. The cells were stained in a stepwise procedure: (1) mouse anti-human-IgM (Becton Dickinson), (2) goat anti-mouse-PE-Texas-Red (Invitrogen), (3) goat anti-human-IgG-biotin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), goat anti-human-IgA-biotin (Invitrogen), (4) Streptavidin-APC (Becton Dickinson), mouse anti-human-CD27-FITC (Becton Dickinson), rabbit anti-human-IgD-PE (Dako), mouse anti-human-CD38-PerCPCy5.5 (Becton Dickinson), mouse anti-human-CD5-PECy7 (Becton Dickinson), and mouse anti-human-CD19-APCCy7 (Becton Dickinson) and sorted, using a FACSVantage Diva (Becton Dickinson) to a purity of >95%. Notably, plasmablasts and plasmacells were excluded from the analysis by separating cells with very high CD38 expression (plasmablast and plasmacells) from cells with high CD38 expression (CC, CB). The freshly isolated cells were all lysed in Trizol (Invitrogen). Details of which markers that were used to separate the different B-cell populations are shown in Table II.

TABLE II. Sorting Parameters for Each Nonmalignant B-Cell Population

Population	Designation	CD19	CD5	IgD	IgM	CD38	CD27	IgA/IgG
Naïve B-cells	N	+	−	+	+	−	−	n.d[1]
Centroblast	CB	+	−	−	−	+	n.d.[1]	−
Centrocyte	CC	+	−	−	−	+	n.d.[1]	+
Memory B-cells	M	+	−	−	−	−	+	+

[1] n.d - not determined

Isolation of mRNA and sample preparation

Briefly, total RNA was isolated from sorted cells (malignant and nonmalignant) lysed in Trizol (Invitrogen), and the RNA quality was controlled. The RNA was then subjected to *in vitro* transcription, according to the Two-cycle Eukaryotic Targeting Labeling assay protocol (Affymetrix, Inc., Santa Clara, CA). The labeled cRNA was hybridized to the Human Genome U133 Plus 2.0 arrays containing ~50,000 transcripts (Affymetrix). This complete procedure is described in detail previously [17,20]. All arrays included in the study passed the initial quality control, including comparison of scaling factors, background, noise, and GAPDH expression with threshold values defined by Affymetrix.

Gene expression analyses

For each probe set, the expression level is given as a signal value in the GeneChip Operating Software (GCOS) 1.4 (Affymetrix). The signals on each array were scaled to an average target value of 100 (MAS5). These normalized signal values were imported into Gene Spring GX 10 (Agilent) and logarithmic transformed.

Identification of present transcripts. All transcripts on the array (referred to as "Array total transcripts") were filtered to have a present call (as defined by the algorithm in GCOS 1.4) in all three samples in at least one of the four nonmalignant B-cell populations N, CC, CB, and M. This resulted in 23,019 transcripts, from now on referred to as "B-cell present transcripts". Furthermore, the "Array total transcripts" were filtered to be present in at least two of 78 BCL samples resulting in 39,555 transcripts referred to as "BCL present transcripts." These lists of transcripts were further analyzed as described below.

Identification of transcription factors. In addition, the ENSEMBL database (2008-05-30) was filtered for all genes identifications (ID) annotated as "transcription factor activity" resulting in 1859 genes. These genes corresponded to 2597 transcripts available on Affymetrix Human Genome Plus 2.0 arrays since some of the TF genes were represented by more than one transcript. To this list, we added three Affymetrix transcripts encoding Sox11 resulting in a total of 2600 transcripts encoding TFs (referred to as "Array total TF transcripts"). These transcripts were further filtered to be present in at least two of the 78 BCL samples and 1886 transcripts hereafter referred to as "BCL present TFs transcripts," passed this filter and were further analyzed. To characterize the genes corresponding to the identified transcripts, annotation in Affymetrix were used. An overview of the different analyzes performed in this study is shown in Fig. 1.

ANOVA. To identify transcripts with a unique expression profile in the different BCL entities, the "BCL present transcripts" as well as the "BCL present TF transcripts" were analyzed using one-way ANOVA test (F-test) followed by Tukey's Honestly Significant Difference (HSD) post hoc test, with the false discovery rate (FDR) controlled by the Benjamini and Hochberg method [21] ($q < 0.05$). Intersections of transcripts significantly separating one group from all other groups were identified. However, for some entities, it was not possible to find transcripts that differed significantly thus these entities (DLBCL and MCL subgroups, respectively) were grouped together. The analysis was performed in Gene Spring (Agilent).

Unsupervised subgroup identification. Variance filtration was applied on the "B-cell present transcript" to explore unsupervised clustering of different entities of BCLs. The filtration process was terminated as soon as one group, defined as having all or all but one of the nearest neighbors within the same group, was formed, and the group was removed. Filtration was then performed again for the remaining

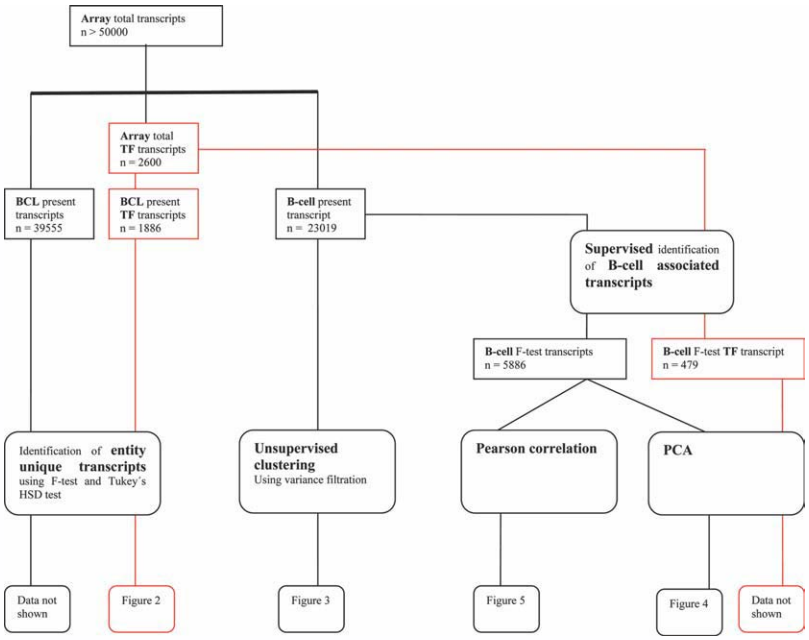

Figure 1. Schematic figure of the different analyses performed. Four major analyses of the collected gene expression data were performed. In the figure, the designated name and number of filtered transcripts are presented, as well as the characteristics of the different analyses performed. Red lines indicate analyses based on transcripts encoding transcription factors (as defined in ENSEMBL–Material and Methods).

samples. Since variance filtration depends on the samples present in the analysis, all "B-cell present transcripts" were used for all filtrations. This procedure was iterated until no more groups could be separated. The analysis was performed using Qlucore Omics Explorer 2.0 (Qlucore AB, Lund, Sweden).

Interaction analysis. To analyze for interactions between identified TFs and potential down-stream genes, the lists of uniquely expressed TFs and genes associated with each biological group, as determined by unsupervised clustering, were imported to the Ingenuity Pathway Analysis tool (Ingenuity® Systems, Mountain view, CA, www.ingenuity.com) and a network analysis performed. Interactions defined by IPA as expression, transcription, and activation were further investigated in the literature to identify specific interactions.

Supervised identification of differentially expressed transcripts. "B-cell present transcripts" and "Array total TF transcripts" were filtered to distinguish the three nonmalignant B-cell groups [N, GC B-cells (including CB and CC) and M], using multi group comparison (F-test), controlling for false discovery rate by the Benjamini and Hochberg method [21] ($P < 0.003$, $q < 0.01$, respectively, $P < 0.003$, $q < 0.05$) The resulting lists, "B-cell F-test transcripts" and "B-cell F-test TF transcripts" contained 5886 and 479 transcripts, respectively. To validate these analyses, permutation tests, where the subgroup-labels were randomly assigned, was performed repetitively and resulted in insignificant number of genes confirming the statistic significance of the above identified transcripts. The analysis was performed using Qlucore Omics Explorer 2.0 (Qlucore AB).

Correlation analysis. To enable comparison between samples in the nonmalignant group and samples in the malignant group, all samples were normalized to the mean using the elimination factor algorithm in Qlucore Omics Explorer 2.0 (Qlucore AB).

Two different methods were used to analyze the nonmalignant B-cells and the BCL entities ($n = 90$) by supervised analyses; (i) The different populations were visualized by a PCA, and each sample was associated to its nearest neighbor (based on Euclidian distance) using both "B-cell F-test transcripts" and "B-cell F-test TF transcripts", (ii) The Pearson correlation between each entity of malignant and nonmalignant B-cells was calculated based on the "B-cell F-test transcripts."

Results

Analysis of purified B-cell lymphomas

The proportion of tumor cells in each complex BCL sample varied; CLL (69 to 97%), DLBCL-GC (20 to 80%), DLBCL-nonGC (20 to 90%), DLBCL-tr (60 to 80%), FL (35 to 80%), HCL (61 to 97%), L-MCL (63 to 93%), MCL (59 to 97%), clearly reflecting the need for enrichment. By using flow-cytometry based cell-sorting, the clonal B-cell population was increased from a heterogeneous 20–97% to a homogenous >97%, allowing a quality assured interpretation of gene expression data relevant for the tumor cells. This procedure dramatically improved the analytical resolution in this study and enabled identification of gene expression differences in tumor cells.

B-cell lymphoma entities express unique sets of transcription factors

Since TFs orchestrate a large part of all cellular processes and functions, we hypothesized that these factors can likely be used to distinguish a unique biology associated with each BCL entity. Consequently, we sought to specifically identify TFs that can be uniquely linked to different BCLs. We generated a list of 2600 transcripts corresponding to known TFs based on annotations from the ENSEMBL database, which we designated "Array total TF transcripts". To identify TFs with a unique expression profile in each of the BCL entities, an F-test followed by Tukey HSD post hoc test ($q = 0.05$) was performed and transcripts significantly deregulated between CLL, DLBCL (including DLBCL-GC, DLBCL-nonGC, and DLBCL-tr samples), FL, HCL, and MCL (including MCL and L-MCL samples) could be identified.

The number of unique TF transcripts, and corresponding characterized genes, varied between entities, such as FL ($n = 10$, corresponding to seven genes), DLBCL ($n = 11$, corresponding to 10 genes), MCL ($n = 19$, corresponding to 10 genes), CLL ($n = 33$, corresponding to 21 genes), and HCL ($n = 169$, corresponding to 82 genes) (Table III and Fig. 2a–e). This analysis revealed that HCL displays the largest number of distinct transcripts, clearly distinguishing it from the other BCL entities. MCL could not be separated from L-MCL, nor could the different DLBCL subgroups be distinguished based on TF expression profiles.

The relative mRNA expression of all unique TF transcripts in BCLs and nonmalignant B-cell populations are

TABLE III. Unique Characterized TF Genes for Each B-Cell Lymphoma Group[1]

Gene symbol[2]	Gene symbol
FL	**CLL**
ZNF496*	EBF1*
TSHZ1*	LASS4*
CCRN4L*	EGR*1
HOPX	TRERF1*
STAT5B	FOS*
FOXO1	ZNF93*
TSC22D4	E2F3*
	SOX4*
DLBCL	BHLHB3*
EP300*	LOC641518
MLL*	PHTF1
TSC22D2*	LEF1
KLF2	C21orf41
PHF1	RXRA
ZNF274	THRA
CREBBP	SMAD4
ZNF238	YY1
PTTG1	ARNT
PA2G4	PRDM2
	PBX3
HCL[3]	HIRA
TCF4*	
TAF4B*	**MCL/L-MCL**
ETS1*	NR3C1*
MYBL2*	SEMA4A*
TCF7L2	TCF25
MAF	MEF2C
GAS7	MAFB
MYF6	SALL2
SIX3	GTF2IRD1
HOPX	IRF4
PRDM16	SOX8
TSHZ3	SOX11
ETV5	
TBX15	
SOX5	
ASCL2	
SOX4	
PPARG	

[1] This table is an assortment of all TF transcripts present in Supplementary material (Table S1)

[2] * Equals low expression of the transcript in the specified group.

[3] The characterized genes corresponding to the 50 transcripts (of total 169) with best *q*-value.

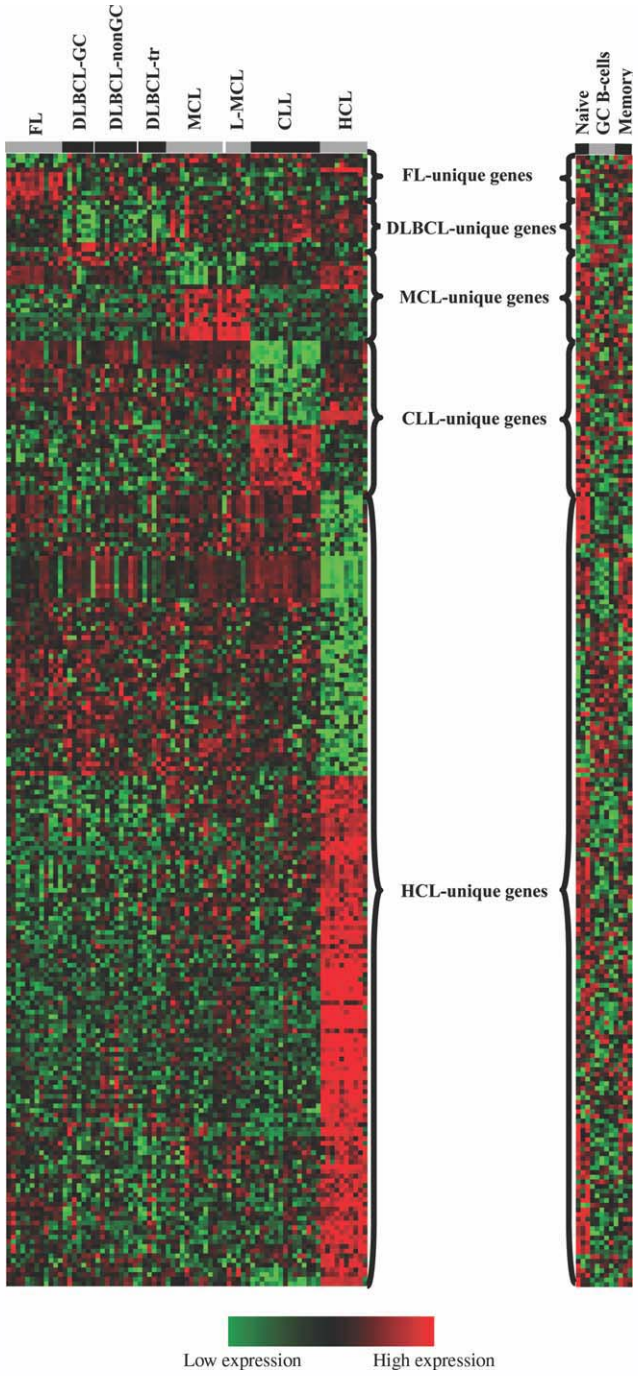

Figure 2. Heat map representing sets of unique transcription factors for each B-cell lymphoma subtype. The relative expression of each transcription factor, filtered to have unique profiles in different B-cell lymphoma entities, is visualized in malignant entities (n = 5, including 78 samples) as well as in the nonmalignant populations (n = 3, including 12 samples). The corresponding gene names are shown in Supplementary material (Table SI). Red color indicates up regulation, green color indicates down regulation, whereas black color indicates no change. Notable these heat maps can only be used for qualitative assessment of the expression of each gene over the samples.

shown in Fig. 2. Of note, most of the transcripts, identified to be deregulated in BCLs, were not shown to be differentially regulated during normal B-cell differentiation. Among these TFs (Fig. 2, Table III and Table SI), tumor suppressor genes or TFs regulating tumor suppressors were identified for all of the entities including expression of *FOXO1* [22] in FL cells, *PA2G4* [23] in DLBCL cells, *SOX11* [24] in MCL cells, and *PRDM2* [25] in CLL. Furthermore, *SOX4* and *EGR1*, known depending on tumor type, to act both as tumor suppressors [26–29] and oncogenes [30,31] showed lower expression in CLL cells. Finally, HCL cells had a high expression of *SOX4*, and also a low expression of *FOXP1* [32]. These results suggest a functional role for the TFs related to the malignant transformation. Of note, *IRF-4*, expressed in high levels in DLBCL-nonGC [33] was for the first time demonstrated to be active in MCL. Furthermore, HCL expressed a high number (n = 169) of unique TF transcripts, where *MAF* [34] may direct HCL toward a myeloid cell differentiation [35,36].

Unsupervised clustering of B-cell lymphomas and comparison to diagnostic subgroups

To verify that the diagnostic subgroups are biologically distinct when analyzing purified tumor cells, an unsupervised clustering was performed. Our results demonstrated that the majority of samples clearly associated with their diagnostic subgroup (Fig. 3). Remarkably, we observed 11,233 transcripts that clearly separated group A, including all 10 HCL samples (Fig. 3a), and as in the TF-analysis, HCL demonstrated a distinct transcriptional profile. After excluding group A, a new filtration resulted in 253 transcripts that separated the data set into two distinct groups (Fig. 3b). Group B1 included all 15 CLL samples, but also 1/12 FL and 1/6 L-MCL sample and

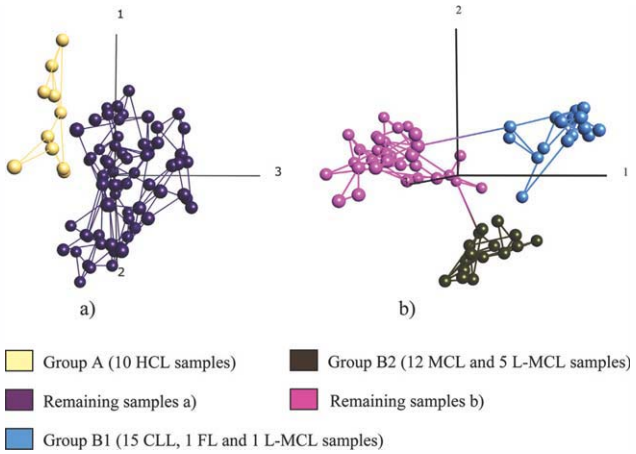

Figure 3. Subgrouping of B-cell lymphomas through variance filtration. PCA plots are used to visualize the results from the unsupervised variance filtration used to group different BCL specimens. The two nearest neighbors for each sample is also visualized in the plots. Transcripts are filtered for high variance across samples, and when a group of samples have one or less nearest-neighbors outside the group it is defined as a distinct group and removed from the analysis. (a) Already at 11,233 transcripts, group A, containing 10/10 HCL samples, was formed. The visualization was made for 7849 transcripts to improve resolution in the picture, with the variance in the three major principal components being; 1 = 15%, 2 = 11%, and 3 = 7%. (b) The remaining samples from (a) was further filtered, and 253 transcripts were used to separate the next two groups, with the variance in the three major principal components being; 1 = 25%, 2 = 10%, and 3 = 7%. Group B1 included all 15 CLL samples, but also 1/12 FL and 1/6 L-MCL sample and group B2 included all 12 MCL samples and 5/6 L-MCL samples. The remaining samples in (b) could not be further separated and consisted of all 23 DLBCL samples and 11/12 FL.

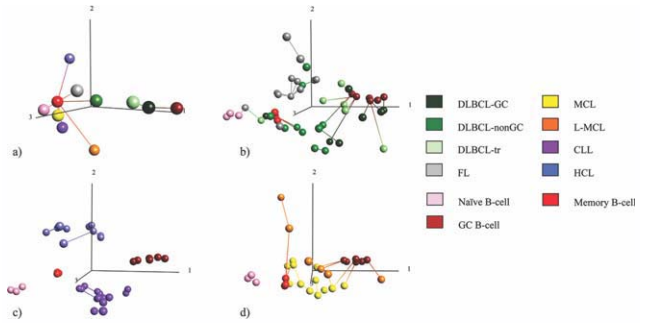

Figure 4. PCA representation of B-cell lymphoma entities and nonmalignant B-cells based on B-cell associated transcripts. PCA plots were used to visualize the relation between different lymphoma entities and nonmalignant B-cell populations. The nearest neighbor for each sample is also visualized in the plots. The analysis is based on 5,886 transcripts (B-cell F-test transcript) filtered by multi group comparison ($P = 0.003$, $q = 0.01$) to maximize the separation of the nonmalignant B-cell populations centroblasts and centrocytes (GC-B-cells, $n = 6$), naïve B-cells (N, $n = 3$) and memory B-cells (M, $n = 3$). (a) The mean values for all populations are visualized in the PCA plot, with the variance in the three major principal components being; 1 = 47%, 2 = 14%, and 3 = 12%. (b) All samples in the three nonmalignant B-cell populations and all FL, DLBCL-GC, DLBCL-nonGC, and DLBCL-tr samples are visualized, with the variance in the three major principal components being; 1 = 31%, 2 = 9%, and 3 = 7%. (c) All samples in the three nonmalignant B-cell populations and all CLL and HCL samples are visualized, with the variance in the three major principal components being; 1 = 26%, 2 = 14%, and 3 = 11%, (d) All samples in the three nonmalignant B-cell populations and all L-MCL and MCL samples are visualized with the variance in the three major principal components being; 1 = 34%, 2 = 11%, and 3 = 11%.

a

	DLBCL-nonGC	DLBCL-tr	FL	MCL	L-MCL	CLL	HCL	GC B	Memory	Naïve
DLBCL-GC	0.47	0.69	0.10	-0.39	-0.37	-0.57	-0.56	0.77	-0.72	-0.70
DLBCL-nonGC		0.37	0.16	-0.25	-0.44	-0.36	-0.44	0.32	-0.30	-0.29
DLBCL-tr			0.29	-0.45	-0.41	-0.55	-0.54	0.66	-0.60	-0.61
FL				-0.31	-0.44	-0.25	-0.22	0.13	-0.02	-0.18
MCL					0.36	0.12	-0.02	-0.45	0.34	0.47
L-MCL						0.19	0.00	-0.26	0.15	0.30
CLL							0.12	-0.44	0.40	0.41
HCL								-0.45	0.48	0.36
GC B									-0.89	-0.94
Memory										0.69

Pearsson correlation (-1) – 0
Pearsson correlation 0 – 0.33
Pearsson correlation 0.33 – 0.67
Pearsson correlation 0.67 – 1

b

Population 1	*Population 2*	*PCC*
DLBCL-GC	GC B-cells	0.77
DLBCL-GC	DLBCL-tr	0.69
Memory B-cells	Naïve B-cells	0.69
DLBCL-tr	GC B-cells	0.66
HCL	Memory B-cells	0.48
DLBCL-GC	DLBCL-nonGC	0.47
MCL	Naïve B-cells	0.47
CLL	Naïve B-cells	0.41
CLL	Memory B-cells	0.40
DLBCL-nonGC	DLBCL-tr	0.37
MCL	L-MCL	0.36
HCL	Naïve B-cells	0.36
MCL	Memory B-cells	0.34

Figure 5. Pearson correlation coefficients between different entities of malignant and nonmalignant B-cells. (a) Pearson correlation coefficient (PCC) are calculated using the mean value for the 5,886 transcripts (B-cell F-test transcripts) filtered by F-test ($P = 0.003$, $q = 0.01$) to maximize the separation of the nonmalignant B-cell populations ($n = 3$). PCC are calculated for all malignant and nonmalignant B-cell entities. (b) The groups with the highest PCC values are listed.

group B2 included all 12 MCL samples and 5/6 L-MCL samples the remaining samples consitute 11/12 FL samples, all seven DLBCL-GC samples, all 10 DLBCL-nonGC samples, and all six DLBCL-tr samples that could not be further separated. The unsupervised analysis showed that HCL, CLL, MCL, and DLBCL/FL are recognized as biological groups and that DLBCL and FL cannot be separated.

The unsupervised clustering indicates that differences in expression of TFs, influence the global transcription profile, since the number of uniquely expressed TFs correlates to the total number of genes used to separate each B-cell lymphoma subgroup. This association is further supported by the finding that genes down-stream of the unique TFs were identified in the same BCL subgroup. These associations were based on an analysis where unique TFs were compared with genes associated with the specific subtypes in the unsupervised analysis, as assessed by identifying relations using IPA (Ingenuity® Systems). For example, in HCL (i) *PPARγ* are involved in the regulation of *CAR2* [37] and *KLF4* [38], (ii) *SIX3* is shown to increase the expression of *CCND1* [39] and (iii) *TBX21* is known to increase the expression of *CXCR3* [40]. In CLL genes verified to affect *CCND1* are *LEF1*, showing a high expression, and *E2F3*, showing a low expression [41,42]. However, more than 11 unique TF transcripts, were needed to affect the global gene expression as demonstrated by the inability to separate FL and DLBCL.

Supervised clustering using B-cell associated transcripts

FL and DLBCL are clinically identified as different diseases [16], despite this, the unsupervised clustering could not separate them. Therefore, we explored the separation of all BCL entities based on B-cell associated genes, which forms the basis of the current diagnostic strategy. These BCL entities were analyzed using 5,886 transcripts ("B-cell F-test transcripts") significantly separating the B-cell populations (N, GC B-cells and M) ($q < 0.01$).

To ensure the biological relevance when analyzing the relation between different malignant and nonmalignant B-cell populations, two different statistical methods were used, (i) PCA in combination with nearest neighbor (Fig. 4) and (ii) Pearson correlation analysis (Fig. 5). Notable, in BCLs, the gene expression of the identified "B-cell F-test transcripts" are within the same range as normal B-cells, as illustrated by PCA plots (Fig. 4a–d). This is of interest, since it was expected that BCLs would display a more

extreme expression of genes involved in for example proliferation. In addition, memory B-cells and naïve B-cells are known to have different functions, however, the strong correlation between them suggest them to be transcriptionally similar (Figs. 4 and 5).

By using the "B-cell F-test transcripts", we were able to separate the three different subgroups of DLBCL, as well as FLs (Figs. 4 and 5). Although, pure lymphoma cells were analyzed, and, thus, the intratumoral heterogeneity was significantly reduced, a profound heterogeneity in the different DLBCL-tumor specimens was still observed. This heterogeneity is likely due to translocations and random mutations in genes other than immunoglobulin genes [43]. However, the DLBCL-tr and DLBCL-GC showed more similar gene expression compared with DLBCL-nonGC and correlated closely (Figs. 4a,b and 5a,b). Notably, DLBCL-tr and DLBCL-GC evolve through different mechanisms but some DLBCL-GC may have passed through an undiagnosed primary FL and may thus be misdiagnosed. These two subgroups also demonstrate a closer relation to GC B-cells, their suggested precursor cell, than to the resting B-cells (M and N), as previously reported [44,45].

CLL and HCL, as well as L-MCL and MCL show a closer association to the resting B-cell populations (M and N), especially to memory B-cells, than to the proliferating GC B-cells (Fig. 4a). However, separation of L-MCL and MCL were difficult also using these B-cell associated genes (Figs 4a,d), underlining the clinical paradigm that these entities should be considered as a single disease [16]. In addition, we demonstrated that CLL could not be separated based on IGHV mutational status. This result is in agreement with previous studies identifying only a limited number of genes to be differentially regulated between IGHV mutated and unmutated CLL [46,47].

Discussion

As a B-cell undergoes malignant transformation, distinct phenotypic characteristics, also associated with normal B-cell differentiation, are often conserved [48]. Consequently, the classification of BCLs has historically been associated with the cell of origin [16]. Therefore, previous studies have analyzed lymphomas according to origin to understand the functional and biological context of each specific lymphoma entity [3,45,49–52]. However, it is clear that many differences related to the diverse clinical outcome among different B-cell lymphomas cannot be related to B-cell associated features but must be explained by specific features related to each unique lymphoma subgroup.

Historically, most microarray studies have assessed the transcriptome from whole tissue samples, comprising various tumor cells, nonmalignant infiltrating cells, and stromal cells. In the case of FL, it has been shown that the overall survival is to a great extent determined by the interplay between tumor cells and the surrounding bystander cells [6–8]. In the case of MCL and DLBCL, on the other hand, the clinical outcome is mainly determined by the tumor cell alone [3,5]. Thus, for most analyses, as when analyzing BCLs for tumor cell-associated antigens and when correlating BCL cells to their normal counterparts, it is of major importance that the mRNA analyzed are from homogenous cell populations. In this study, we analyzed a set of eight flow-cytometry sorted populations of BCLs (CLL, DLBCL-GC, DLBCL-tr, DLBCL-nonGC, FL, HCL, L-MCL, and MCL) and four populations of nonmalignant tonsil B-cells from different predefined stages of differentiation (N, CB, CC, M). Thus, as homogenous cell populations were used, results can be related to difference in cellular gene expression in contrast to variation in tumor tissue content.

TFs have not been a central focus of drug development, but recent advances suggest them to be worthwhile targets for cancer therapy [12,53,54]. In this study TFs, not necessarily linked to B-cell differentiation but to distinct biological features of each BCL subgroup and potentially responsible for the malignant transformation were identified. Most of these identified TFs did not show any differential regulation in normal B-cell differentiation, probably due to the fact that genes separating nonmalignant B-cells in many cases are shared between more than one B-cell lymphoma subtype. Furthermore, TFs with direct or indirect tumor suppressor function were found in each BCL subgroup as well as other individual genes, explaining functional features previously reported to be associated with the specific subgroup. Amongst others, HCL, shown to be a distinct group in all analyses performed, has, for example previously been shown to occupy phagocytic capacity [55,56]. This is reflected in the distinct gene expression profile observed for HCL cells as compared with other BCLs and nonmalignant B-cells using both a focused list of TFs and unselected genes.

For several of the HCL and CLL-associated TFs, down-stream targets are found among the unselected genes identified in the unsupervised clustering. Thus, not only is the number of transcripts separating different lymphoma subgroups related to the number of unique TFs but also specific examples of down-stream genes are identified. However, for DLBCLs and FLs where $\leq$ 11 transcripts coding for TFs could be identified, the unsupervised clustering was unable to distinguish these subpopulations. The inability to subgroup DLBCL and FL indicated that there are a certain number of TF genes that are needed to affect the global gene expression to that extent that unsupervised clustering can successfully identify distinct groups. Despite this, it is clear that FL and DLBCL have both morphological and clinical differences although they are both related to the GC B-cell stage. However, using genes associated with normal B-cell differentiation, we were able to separate also FL and the different molecular groups of DLBCL, including DLBCL-tr, DLBCL-nonGC and DLBCL-GC from each other. This suggests that these BCLs differ in genes important for normal B-cells differentiation, as previously seen for DLBCL [3,57,58].

FL, like DLBCL, is known to originate from GC B-cells [59,60], but gene expression profiles of FL were surprisingly dissimilar to GC B-cells in our study. This might be explained by the t(14;18) translocation in FL leading to an over expression of BCL2 [61], which regulates other pathways and thereby changing the expression profile. Lymphomas with this translocation tend to have an indolent growing pattern [16,62], likely to result in a distinct gene expression profile.

Our correlation analysis showed that HCL and CLL samples were, in agreement with the unsupervised clustering and the high number of uniquely expressed TFs, distinct and homogenous populations having a transcriptional profile enabling separation in a different dimension, compared with the nonmalignant B-cells (Figs. 4a,c and 5a). In addition, in agreement with the unsupervised clustering, leukemic and nodal MCL are clinically regarded as one disease, therefore, a higher Pearson correlation coefficient between these subgroups was expected. Potentially, this somewhat low correlation could be explained by the heterogeneity observed within the L-MCL samples in this study (Fig. 4d). Nevertheless, specific genomic changes associated with leukemic dissemination and shorter survival times have been observed previously [63,64]. The TF *IRF-4* known to separate DLBCL-nonGC from DLBCL-GC [57] and to be expressed in a variety of lymphomas of mature B cells

(CLL, MM, DLBCL–nonGC) [57,65,66] here show an high mRNA expression in MCL. However, previous studies have not been able to confirm the IRF-4 protein expression in MCL [67]. The functional role of *IRF-4* in lymphomas has become evident lately although yet not fully elucidated [12]. Studies show an association between polymorphism in the *IRF-4* locus and development of CLL [68] as well as the involvement of the gene in multiple myeloma [69]. The functional effect of *IRF-4* in MCL have to be further assessed in future studies.

We showed that HCLs have a gene expression profile distinct from all other BCLs. This gene profile could not easily be assigned to any of the nonmalignant B-cell populations but were most closely related to memory B-cells (Fig. 4a). This is in agreements with previous studies where similarities to both memory B-cells [34] and splenic marginal zone B-cells [70–73] has been observed. The cause of the distinct gene expression profile of HCL cells could be either due to an onset of a unique signaling pathways compared with the other BCLs which is indicated by the high expression of *MAF* suggested to change the expression profile toward a myeloid differentiation [35,36], or a distinct cell of origin, potentially a splenic B-cell, which remains to be determined.

In summary, we have identified TFs that uniquely identify eight different B-cell lymphoma subtypes, using highly purified BCLs, which enabled the specific analysis of the gene expression of tumor cells. The identified TFs partly also explain the downstream global and specific gene expression of individual subgroups of BCL. Interestingly, these TFs may be novel functional, diagnostic, prognostic, or therapeutic targets.

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