Reliable subtype classification of diffuse large B-cell lymphoma samples from GELA LNH2003 trials using the Lymph2Cx gene expression assay

Diffuse large B-cell lymphoma (DLBCL) comprises two molecular subgroups originally defined according to gene expression profiling (GEP) of frozen tissue samples: the germinal center B-cell-like (GCB) and activated B-cell-like (ABC) lymphomas, which show major differences in essential biological processes. This classification has a strong prognostic impact and was predictive of response to specific therapies in early phase trials.[1,2] There is a need for a test that can be used to stratify patients' treatment in future clinical trials and that can be translated into a routine lab procedure as a companion diagnostic test when molecular hallmarks of DLBCL will be used to decide upfront therapy. The Lymphoma/Leukemia Molecular Profiling Project (LLMPP) described a digital gene expression-based assay using NanoString technology (Lymph2Cx) which can be performed using formalin fixed paraffin embedded (FFPE) tissue samples, and which shows a strong correlation with the original cell-of-origin (COO) classification.[3] In a retrospective series of DLBCL patients included in the Groupe d'Etude des Lymphomes de l'Adulte (GELA) clinical trials, we used FFPE tissue samples to compare the COO classification obtained with the Lymph2Cx assay to the gold standard classification, based on Wright's predictor using Affymetrix data on matched frozen samples. Our findings indicate that this assay, performed in an independent series and a non-LLMPP laboratory, proved to be reliable to classify FFPE samples of a large series of DLBCL.

This study was based on lymphoma tissue samples of patients who had been included in the GELA/LYSA sponsored LNH-2003 program, which enrolled more than 1500 patients into different clinical trials combining rituximab and chemotherapy, or who had been included in the GELA LNH01-5B trial (*Online Supplementary Table S1*). All the patients included in these trials had signed an informed consent to the use of their samples for research, in agreement with French regulations. The histological diagnosis of *de novo* DLBCL had been confirmed by a panel of expert hemato-pathologists. Gene expression profiling (GEP) data from frozen tissues were available for 221 patients. RNA expression had been analyzed with HGU133+2.0 Affymetrix GeneChip arrays. Raw feature normalization and quality check were handled using Bioconductor software (affy, affyQCReport, GCRMA), excluding multi-gene probesets (x). The Affymetrix COO classification into GCB, ABC or Unclassified was made according to Wright's predictor.[4,5] Primary mediastinal B-cell lymphomas (PMBL) were identified with hierarchical clustering (complete distance, Ward agglomeration) according to a previously published gene signature, excluding *E2F2* probe sets (which did not fit the PMBL Lymphochip profile) and TCL1A probe sets which induced a strong classification bias.[6] The genetic features of this series of cases, according to the COO, have been recently described.[7] After careful review of the FFPE material, matched FFPE tumor samples were available for RNA extraction for 168 of these 221 patients. The 168 cases included: 64 ABC, 63 GCB, and 26 Unclassified DLBCL, as well as 15 cases identified as PMBL, diagnosed between March 2002 and April 2011. Total RNA were extracted from FFPE tumor samples with a fully automated method (Siemens Healthcare Diagnostics), using 1-3 10 μm tissue scrolls of 0.4±0.3 cm^2, 2-12 years after diagnosis.[8] Fifty ng RNA were hybridized to the Lymph2Cx CodeSet, using high sensitivity setting and analyzed with the Nanostring nCounter Analyzer (Generation 2; maximum resolution: 555 FOV). We only used 30 ng RNA for 4 samples for which there was no more RNA available. Digital counts were used to calculate the normalization and linear predictor score (LPS), ABC likelihood and "raw" subgroup prediction. It has been recognized that lot-to-lot variability introduces bias between lots and that, in one study, this bias was reduced from 52 points to 26 points (on a scale of approximately 4000 units) when synthetic reference oligonucleotides were used to correct for hybridization differences.[9] In the current study, the LPS was adjusted using the same method with counts from reference oligonucleotides on the new code set; this adjustment reduced the number of unclassified samples and was used in the analyses that follow.

Despite heterogeneity in fixatives, the experiments were successful in 157 of 168 (93%) FFPE samples (Figure 1). For 11 cases, the normalization score was below the threshold (20) required for reproducible results, and the experiments were considered as failed. These 11 cases included 2 cases with low RNA amounts, 2 cases extracted from AFA (acid acetic/formol/alcohol) blocks, and 5 cases with unknown fixative. Still, it is noteworthy that successful analysis was achieved in tumor samples fixed in AFA (n=13), Bouin's fixative (n= 4), or unspecified fixative (n=44) (Figure 1) and in 2 cases with low RNA amounts (30 ng), showing that the assay was robust even when different fixatives were used. In 5 cases, RNA was extracted twice or 3 times from the same block and the classification was concordant for each of these extractions. In 2 cases, the second RNA extraction shifted the classification from GCB to Unclassified. The histological control of these 2 samples showed that the paraffin block had been exhausted by the first extraction and no longer contained a significant amount of tumor cells. In one case, the first RNA extraction (with low RNA content)

Table 1. Comparison of Affymetrix classification and Lymph2Cx classification in 144 diffuse large B-cell lymphoma samples.

		Lymph2Cx assay			
		GCB	Unclassified	ABC	Total
Affymetrix COO	GCB	**53**	7	1	61
	Unclassified	5	**9**	9	23
	ABC	0	1	**59**	60
Total		58	17	69	

Concordant classification with both tests in bold.

failed whereas the second one proved successful.

Lymph2Cx data were obtained for 157 patients: 13 with primary mediastinal B-cell lymphoma (PMBL) and 144 with DLBCL. PMBL samples had been included in the study because patients with this specific subtype of lymphoma had been included in GELA LNH2003 DLBCL trials. However, it is now well recognized that this lymphoma subtype is a distinct entity with specific biological features.[10] Therefore, we removed these PMBL cases from comparisons of the Lymph2Cx assay to Affymetrix classification. The 144 DLBCL samples were classified as ABC (n=69), Unclassified (n=17), and GCB (n=58) with the Lymph2Cx assay (Figure 2). As shown in Table 1, in our hands, the performances of the Lymph2Cx classifier were very close to that of the validation cohort described by Scott *et al.*[3] When the analysis was restricted to Affymetrix GCB or ABC cases, the Affymetrix and Lymph2Cx classifications were concordant in 92.6% (112 of 121) of the cases (91.4% in Scott *et al.*), the samples were Unclassified in 6.6.% (8 of 121) (6.9% in Scott *et al.*), or misclassified in 0.8% (1 of 121) (1.7% in Scott *et al.*). When considering all 3 categories, GCB, ABC and Unclassified, the classifications were concordant in 84% (121 of 144) of the cases (81% in Scott *et al.*), the samples moved from a definitive subtype to Unclassified (or *vice versa*) in 15.3% (22 of 144) (17.6% in Scott *et al.*), and were misclassified in 0.7% (1 of 144) (1.5% in Scott *et al.*).

The clinical characteristics of the 144 DLBCL cases studied are described in *Online Supplementary Table S2*. We tested the association of the Affymetrix classification, the Nanostring Lymph2Cx classification and the International Prognostic Index (IPI) with outcome: progression-free survival (PFS) and overall survival (OS), in this series of patients. The median follow up was 41.4 months. The IPI was strongly associated with survival in this series of patients (*Online Supplementary Table S3* and *Online Supplementary Figure S1*). In this series of patients, which were heterogeneous regarding the IPI and treatment regimen, the Lymph2Cx classification was not significantly associated with PFS [Hazard Ratio (HR) ABC *vs.* GCB=1.43 (0.77-2.67), log-rank score test *P*=0.26] or OS [HR ABC *vs.* GC=1.55 (0.74-3.22) log-rank score test *P*=0.24] and Affymetrix COO showed a stronger association with higher HRs but without reaching significance.

Our results indicate that the Lymph2Cx classifier on the NanoString technology is remarkably robust, since the concordance with Affymetrix classification proved similar to that of the initial validation study, despite using limited amounts of RNA, slightly different machine setting and FOV parameters and the experiments being performed in an independent lab. It is interesting to note that all but one of the ABC cases were called as ABC by the Lymph2Cx assay. Nine Affymetrix Unclassified samples were also identified as ABC by the Lymph2Cx assay. The immunohistochemical analysis of these samples showed IRF4 staining (7 of 7 cases with available data). There was one misclassification, which might correspond to a frozen sample swap, since the FFPE block immunophenotype was CD10 negative, BCL6 negative, IRF4 positive and FOXP1 positive (*Online Supplementary Table S4*). The major source of discrepancy between the two assays resulted from biopsies, with LPS scores close to the thresholds, shifting between definitive COO subtypes and the Unclassified category. These "intermediate" scores might correspond to samples with low tumor content (as previously reported by Scott *et al.* and observed in 2 cases for which we performed re-extraction and a second analysis), lymphomas with a particular immune infiltrate, or a true "third" DLBCL subtype that has yet to be identified.

Despite the high concordance between the two assays, in this study, the Lymph2Cx assay proved marginally less predictive than the Affymetrix classification for clinical outcome. This may be related to the fact that some cases with a favorable prognosis were "lost" by a shift from

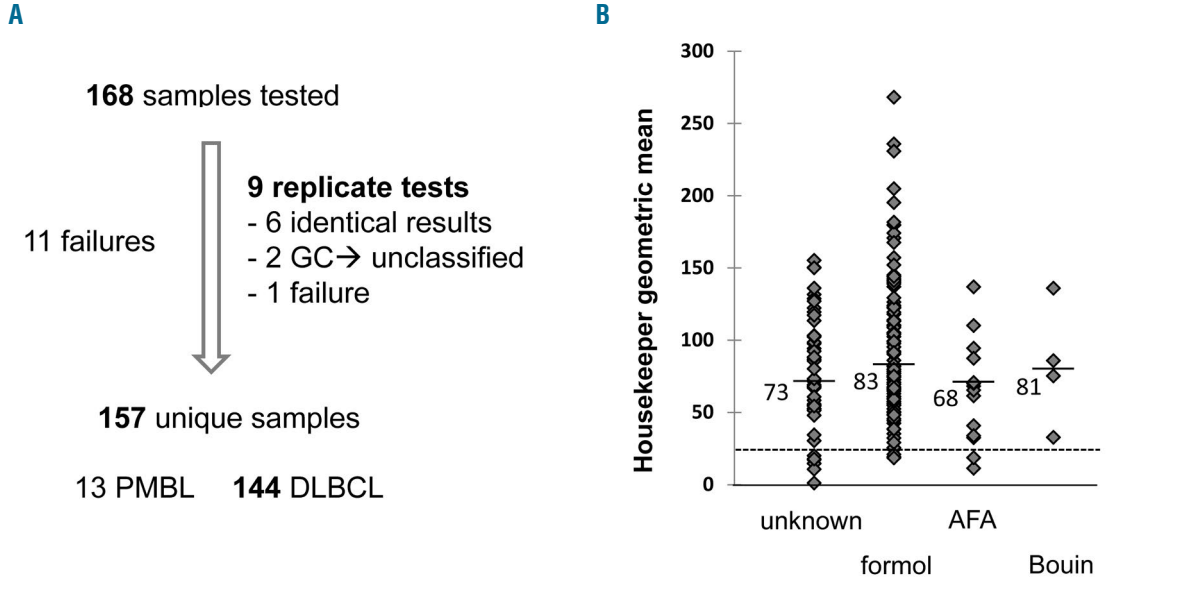

Figure 1. Normalization across various fixatives. (A) Flow chart of the study. (B) The geometric mean of housekeeper digital counts is used to normalize the data, and the threshold for considering the sample to have passed quality control was set to 20 (dotted line). Lymphoma samples were fixed in formalin (n= 99, 3 failures), acid acetic / formalin (n=15, 2 failures), Bouin's fixative (n= 4), or unspecified fixative (n= 50, 6 failures). The mean value for each group is indicated on the graph. GC: germinal center; PMBL: primary mediastinal B-cell lymphoma; DLBCL: diffuse large B-cell lymphoma; AFA: acid acetic/formol/alcohol blocks.

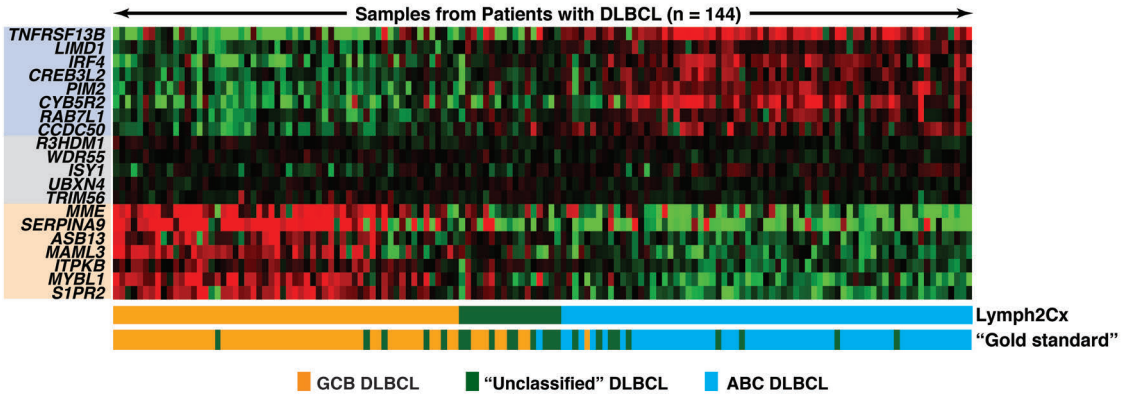

Figure 2. Normalized expression of the Lymph2Cx genes in the 144 diffuse large B-cell lymphoma (DLBCL) samples. The Lymph2Cx model is shown in the form of a gene expression heatmap ordered left to right according to the assay score. The 20 genes that contribute to the model are shown on the left, with the top 8 genes being over-expressed in activated B-cell-like (ABC) lymphomas, the middle 5 genes being housekeeping genes, and the lower 7 genes being over-expressed in germinal center B-cell-like (GCB) lymphomas. The cell-of-origin assignments are shown for the assay (upper lane) and the gold standard method using the Affymetrix data on frozen samples (lower lane).

GCB to Unclassified (n=7), and others shifted from Unclassified (showing a good prognosis) to ABC (n=9). The results recently reported by Scott *et al.*[9] in a large series of patients indicate that the Lymph2Cx has prognostic significance in the population registry-based setting. The lack of Lymph2Cx prognostic significance might also be related to the relatively low number of cases, or to the fact that these cases have been selected from clinical trials and are not population-based. In addition, it is noteworthy that about 43% of the patients received R-ACVBP, which was previously showed to reduce the prognostic impact of the COO classification compared with R-CHOP.[11]

In conclusion, the COO classification of FFPE DLBCL samples is of upmost importance, considering the impact of this classification on intracellular oncogenic signaling pathways and response to specific therapies. The Lymph2Cx assay, performed in an independent series and a non-LLMPP laboratory, proved to be a highly robust assay to classify FFPE samples of a large series of DLBCL.

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