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A novel diagnostic approach for the classification of small B-cell lymphoid neoplasms based on the NanoString platform

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INTRODUCTION

Small B-cell lymphoid neoplasms (SBCLNs) are a heterogeneous group of diseases characterized by malignant clonal proliferation of mature B-cells. However, the classification of SBCLNs remains a challenge, especially in cases where histopathological analysis is unavailable or those with atypical laboratory findings or equivocal pathologic data. In this study, gene expression profiling of 1039 samples from 27 gene expression omnibus (GEO) datasets was first investigated to select highly and differentially expressed genes among SBCLNs. Samples from 57 SBCLN cases and 102 nonmalignant control samples were used to train a classifier using the NanoString platform. The classifier was built by employing a cascade binary classification method based on the random forest algorithm with 35 refined gene signatures. Cases were successively classified as chronic lymphocytic leukemia/small lymphocytic lymphoma, conventional mantle cell lymphoma, follicular lymphoma, leukemic non-nodal mantle cell lymphoma, marginal zone lymphoma, lymphoplasmacytic lymphoma/Waldenström’s macroglobulinemia, and other undetermined. The classifier algorithm was then validated using an independent cohort of 197 patients with SBCLNs. Under the distribution of our validation cohort, the overall sensitivity and specificity of proposed algorithm model were >95%, respectively, for all the cases with tumor cell content greater than 0.72. Combined with additional genetic aberrations including IGH-BCL2 translocation, MYD88 L265P mutation, and BRAF V600E mutation, the optimal sensitivity and specificity were respectively found at 0.88 and 0.98. In conclusion, the established algorithm demonstrated to be an effective and valuable ancillary diagnostic approach for the sub-classification and pathologic investigation of SBCLN in daily practice.

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by 57 SBCLN and 102 nonmalignant control cases, and further validated by an independent cohort of 197 SBCLN cases. In conclusion, we demonstrated a robust, highly accurate, and validated assay for SBCLN distinction using the NanoString platform.

**MATERIALS AND METHODS**

**Patients and samples**

In this study, total 159 cases were retrospectively enrolled in the training cohort, and 197 SBCLN cases were independently recruited in the validation cohort in our center (Tongji Hospital, Wuhan, China). Among the training cohort, 57 subjects were diagnosed as SBCLN, including 7 CLL/SLL, 13 FL, 9 CMCL, 4 leukemic non-nodal MCL (nnMCL), 19 MZL (NMZL, MALT, or SMZL), 5 LPL/WM cases, and 102 non-malignant biopsies from SLL, 13 FL, 9 cMCL, 4 leukemic non-nodal MCL (nnMCL), 19 MZL (NMZL), 32 fresh samples and 165 FFPE samples, which was de

**Table 1.** Patient demographic data and disease characteristics of training and testing cohort.

|                      | Training cohort (n = 57) | Validation cohort (n = 197) |
|----------------------|--------------------------|-----------------------------|
| Age of onset (years) | Median (range) 53 (22–81) | 58 (13–85)                  |
| Gender               | Male 31                  | 118                         |
|                      | Female 26                | 79                          |
| Subtype              |                         |                             |
| CLL/SLL              | 7                        | 14                          |
| cMCL                 | 9                        | 27                          |
| nnMCL                | 4                        | 3                           |
| FL                   | 13                       | 65                          |
| MZL                  | 19                       | 67                          |
| LPL/WM               | 5                        | 17                          |
| Other SBCLNs         | 0                        | 4                           |
| Sample type          |                         |                             |
| FFPE tissue          | 49                       | 166                         |
| Fresh/Frozen sample  | 8                        | 31                          |
| Location             |                         |                             |
| Lymph node/          |                         |                             |
| Waldeyer’s ring      | 35                       | 101                         |
| Peripheral blood/    |                         |                             |
| bone marrow          | 0                        | 8                           |
| Extranodal tissue    | 14                       | 65                          |
| Other                | 8                        | 23                          |
| Tumor cell content   |                         |                             |
| >0.9                 | 57                       | 11                          |
| 0.7–0.9              | 0                        | 84                          |
| 0.3–0.7              | 0                        | 102                         |

**RESULTS**

**Immunophenotypic, cytogenetic, and mutational analyses**

Immunophenotypic, several distinct chromosomal aberrations and somatic mutations were detected and available in all SBCLN cases of the training and validation cohorts at diagnosis. Immunohistochemical (IHC) staining of FFPE tissues and flow cytometry of fresh samples were performed for CD5, CD10 (MMI), Cyclin D1 (CCND1), SOX11, CD38 and other additional biomarkers according to the manufacturer’s protocols. The fluorescence in situ hybridization (FISH) study performed on FFPE tissue sections or fixed cells from cytogenetic cultures was used to interrogate breaks of the loci BCL2, CCND1, MALT1, and IGH (dual color, break apart rearrangement probes) and identify chromosomal abnormalities, including +12 and del (7q), using commercially available probes (Abbott Molecular, USA). Sanger Sequencing was conducted for determining at least 5 hotspot mutations including EZH2 exon18 (Y646), MYD88 exon5 (L265P), BRAF exon15 (V600E), CCKR4 exon2 (S338X), and NOTCH2 exon34 (R2400X). (Supplementary Table S5). A next-generation sequencing (NGS) panel targeted 157 lymphoma-associated genes was also designed to identify other mutations in several samples (Designstudio Sequencing, Illumina, USA) (Supplementary Table S6). The sequencing library was prepared with 20 ng of input DNA per sample and sequenced to 1000x coverage. Generated variants were annotated using AnnoVAR11. Exonic nonsynonymous or splice donor/acceptor site variants with reads ≥20 were initially filtered. Then, variants with a population frequency > 0.0001 in the gnomAD database (gnomAD.broadinstitute.org) were excluded unless they were relevant to lymphoma according to the COSMIC database (https://cancer.sanger.ac.uk/cosmic/).

**Quantitative gene expression study**

Digital expression of samples in the training and validation cohorts was performed with 200 ng of RNA input on the NanoString platform (NanoString Technologies, USA), using the “high sensitivity” setting on the nCounter™ PrepStation and 550 field of view (FOV) on the nCounter™ Analyzer. Normalization of digital gene expression was performed using the geometric mean of the counts of appropriate housekeeping genes for the training cohort and the validation cohort separately. The normalized data were then log2 transformed for further analyses.

**Statistical analysis**

All statistical analyses were conducted by employing R v3.6.2. Differences were evaluated by using Fisher’s exact test for categorical variables. The significance of the co-occurrence or mutual exclusivity was also assessed by utilizing Fisher’s exact test. Nonpaired t tests were used to compare the gene expression level between groups, as appropriate. Unless otherwise specified, a two-tailed P < 0.05 was considered statistically significant for all analyses.

**RESULTS**

Selecting initial candidate genes for classification

In a first step towards developing a classification model, the global GEP of 1039 samples from 27 Gene Expression Omnibus (GEO) datasets (Affymetrix U133 plus 2.0 microarrays, Thermo Fisher Scientific, USA) was initially investigated, including 808 SBCLNs and 231 nonmalignant control cases (Supplementary Tables S7-S8) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi). Raw data of all the samples were normalized together using the 3’ robust multiarray average (3’ RMA) and analyzed by using one-way between-subject analysis of variance (ANOVA). For each subgroup,
differentially expressed genes were selected by one-vs-rest or one-vs-one strategies (Supplementary Table S9). Then, 136 genes were revealed as differentially expressed genes with absolute log2-fold change > 1 and a significant FDR (<0.05). Moreover, 18 other differentially expressed genes referring to associated studies were also included as an essential supplement10,13,16 (Supplementary Table S9). Based on the geNorm algorithm, 13 genes with stable expression were selected as potential housekeeping genes17. Subsequently, these genes were validated in the GEP of 1039 samples using an unsupervised hierarchical clustering approach. Clustering results demonstrated that samples were dominantly distributed in terms of their respective entity and status of sample purification. Compared with the whole gene expression signature, the targeted 154-gene expression signature performed better in clustering samples, because most subgroups had fewer cluster branches and most cluster branches gathered more samples of the same subgroup (Fig. 1) (Supplementary Fig. S1) (Supplementary Table S8). The clustering results indicated that our 154-gene expression signature was feasible for the classification of SBCLN. Thus, a NanoString codeset panel consisting of 154 candidate genes for the classification of SBCLN, along with 13 potential housekeeping genes, was designed for quantitating gene signatures (NanoString Technologies, USA) (Supplementary Table S9). In this panel, capture probes in the codeset were designed to target conserved sequences within all transcripts of each gene.
Table 2. Genes that finally selected in building the molecular classifier.

| Classification | Differentially expressed genes |
|----------------|---------------------------------|
| CLL/SLL        | BTLA, ARHGAP44, ZBTB24, CLNK, CD200 |
| cMCL           | SOX11, PLEKH4B, ZNF711, CCND1a, FAM129C, ABCA6 |
| FL             | BCL2, EML6, ELL3, CTLA4, FCER2, IGFBP3, RGS13, EBFA |
| nnMCL          | CCND1b, ZNF331, PAX5, CNR1 |
| MZL            | FCRLA, HDAC9, MS4A1, SIGLEC6, ZBTB32, BHLHE41 |
| LPL/WM         | CCR2, HOX1, FKBP11, ANK3, ZNF226, MFAPS, MFEBA |
| Additional markers | IGH-BCL2 translocation or t(14;18)(q32;q21) |
| FL             | MYD88 L265P mutation |
| HCL            | BRAF V600E mutation |

CLL/SLL chronic lymphocytic leukemia/small lymphocytic lymphoma, cMCL conventional mantle cell lymphoma, nnMCL leukemic non-nodal mantle cell lymphoma, FL follicular lymphoma, MZL marginal zone lymphoma, LPL/WM lymphoplasmacytic lymphoma/Waldenström’s macroglobulinemia, HCL hairy-cell leukemia. *CCND1 gene was selected as marker in both cMCL and nnMCL.

Quantification of targeted gene expression based on the NanoString platform

Targeted gene expression was thereby quantified in 57 SBCLN and 102 control cases of the training cohort. At the beginning of data processing, 4 genes (ACTB, GAPDH, GUK1, and GUSB) were selected as the final subset of housekeeping genes because the other 9 potential housekeeping genes had low expression levels or a high coefficient of variation across the samples. First, the normalized 154-gene signature of 57 SBCLN cases was analyzed by an unsupervised hierarchical clustering approach. Cases were dominantly clustered into 5 main branches according to their respective subgroup: one was mostly made up of FL cases (13/14, 93%); one included the majority of MZL (16/19, 84.2%) but mingled with 3 nnMCL cases; one was mainly composed of cMCL cases (9/10, 90%); another consisted of all CLL/SLL cases (7/7, 100%); and the last one was exclusively comprised of LPL cases (5/5, 100%) (Fig. 2A). Overall, ~87.7% (50/57) of cases were appropriately clustered, suggesting the feasibility of subsequent development of a classification model using the NanoString platform.

Development of SBCLN classification model

To overcome the difficulty of multiclassification, the model was decomposed into a cascade of one-vs-rest binary classifiers used for multistep discriminations, which was similar to that described by Navarro et al. Each classifier was trained based on the random forest algorithm using the scikit-learn library for the Python programming language (Python Software Foundation, https://www.python.org/) with standard parameters. The order of an SBCLN entity to discriminate was decided according to Davies-Bouldin Index (DBI). Entities with lower DBI were more distinguishable from the other entities and were given priority to discriminate (Supplementary Table S10) (Fig. 2B). In each step, the classifier will determine if a given sample belongs to a designated SBCLN subgroup. Cases were classified as the respective SBCLN if they had a ≥0.5 prediction probability of belonging to the designated entity. The leave-one-out (LOO) cross-validation strategy was used to evaluate the sensitivity and specificity of each classifier. For each classifier, the importance Gini index was used to evaluate the classification ability of all 154 candidate genes, and only the top valuable upregulated genes in discriminating an entity were selected as the final subset of markers to build the one-vs-rest classifier of the entity (Supplementary Table S11). Details of the modeling method are described in the Supplementary Methods. Finally, with a set of 35 genes for the classification in total (Table 2) (Supplementary Fig. S2), our model was trained to successively determine whether a sample belongs to CLL/SLL, cMCL, FL, nnMCL, MZL, LPL/WM, or ultimately annotated as “undetermined cases” (Fig. 2C). CLL/SLL was the first entity to discriminate in the model, with 5 differentially upregulated genes (BTLA, ARHGAP44, ZBTB24, CLNK, and CD200), and was followed by cMCL with 6 markers, FL with 8 markers, nnMCL with 4 markers, MZL with 6 markers, and LPL/WM with 6 markers. Among them, only CCND1 was used two or more times for prediction (both cMCL and nnMCL). The entire model was internally evaluated with the LOO cross-validation strategy. Focusing on SBCLN cases, the overall predictive accuracy was 91.2% (52/57) (Fig. 3A). Five SBCLN cases not classified into their belonging entities, including 1 FL, 1 nnMCL, and 3 MZL cases, were all predicted as “undetermined cases”. This result demonstrated a 100% specificity of our model since no case was misclassified (Supplementary Table S12).

Performance of the model in validation cohort

To determine the validity and reproducibility of the model in an extended and independent dataset, we constructed a validation cohort consisting of 197 SBCLN samples. The tumor cell content ranged from 0.32 to 0.94 (mean 0.67). Digital gene expression data of the validation cohort were independently normalized using 4 final housekeeping genes. Using the 35-gene signature model, 137 cases in total were identified as one of the 6 SBCLN entities, and the other 60 cases were ultimately undetermined (Fig. 4). Among those 137 cases, initial pathological diagnosis was incorrect in 1 FL and 3 MZL cases, and histopathological examination was unavailable in the other 13 cases (Supplementary Table S4), but these 17 cases were all accurately classified into their respective entities by our model. However, misclassification of our model was found in 1 cMCL, 1 FL, 1 MZL, and 1 LPL/WM. Overall, the sensitivity and specificity of our model were 0.68 (133/197) and 0.97 (133/137), respectively (Fig. 3B). The fixation method did not affect the predictive accuracy of model since the differences of predictive accuracy between fresh samples and FFPE samples was not significant (Supplementary Fig. S3).

As expected, the mean tumor cell content in ultimately undetermined cases was significantly lower than that in determined cases (0.55 vs 0.72, p < 0.05), which indicated that tumor cell content had a greater impact on the predictive probability of a case belonging to the respective entity. To make >90% of cases with a tumor cell content no less than the cutoff value have a >0.5 probability of belonging to their respective SBCLN entity, the minimal cutoffs of the tumor cell content were determined to be 0.41 in CLL/SLL, 0.48 in cMCL, 0.72 in FL, 0.78 in nnMCL, 0.68 in MZL, and 0.71 in LPL/WM (Fig. 5A). These results also indicated that our model is more tolerant to lower tumor cell contents in CLL and cMCL samples than in other SBCLN samples. Under the distribution of our validation cohort (Fig. 5B), the overall sensitivity and specificity of our model were both >95% within cases with tumor cell content ≥0.72 (Fig. 5C) (Supplementary Table S13).

We further studied whether combining with other specific chromosomal aberrations and recurrent mutations used in the diagnosis could improve the sensitivity or specificity of our model (Supplementary Table S4). Among 137 cases identified as one of the 6 frequent SBCLN entities, NGS was additionally performed in 4 pathologically misdiagnosed cases, 4 molecular misclassified cases, and 17 other cases with unavailable pathological examination (Fig. 5D). Additional genetic events were considered a combined marker of the model only if it could accurately reclassify ≥2 cases. Combined with EZH2 Y646, MYD88 L265P, and BRAF V600E mutations, the overall sensitivity and specificity were 0.77 (151/197) and 0.98 (151/154), respectively. If only IGH-BCL2 was included as a marker of FL, the overall sensitivity and specificity...
were 0.82 (162/197) and 0.98 (152/155), respectively. If IGH-BCL2, MYD88 L265P, and BRAF V600E were included as supplemental markers of the model, HCL cases could be additionally identified, and the overall sensitivity and specificity could reach 0.88 (173/197) and 0.98 (173/176), respectively (Fig. 3C). More than 95% of cases were correctly classified within cases with tumor cell content ≥ 0.69 (Fig. 5F).

DISCUSSION

The diagnosis of SBCLNs has been notoriously difficult, especially in cases that predominantly had lymphocytosis but did not have lymphadenopathy or accessible tissues for diagnosis. Previous studies have indicated that gene expression signatures could facilitate the diagnosis and classification of SBCLNs, but most of them were largely of binary nature. In this study, using the NanoString platform, we developed a 35-gene signature-based RNA assay to identify major subtypes of SBCLNs, which was validated by an independent cohort. The overall accuracy was >95% within highly purified cases and could be higher if IGH-BCL2, MYD88 L265P, and BRAF V600E were included as supplemental markers. Since this approach requires only very limited laboratory handling and could reliably quantify the expression of a set of genes without interobserver variability, it exhibited potential clinical application prospects as an ancillary method of classification of SBCLN.

There were many difficulties in the development of the model. First, in consideration of the difficulty of an intralaboratory large-scale GEP or RNA-sequencing study, a total of 1039 available global GEP data was analyzed to select differentially expressed markers of the model, HCL cases could be additionally identified, and the overall sensitivity and specificity could reach 0.88 (173/197) and 0.98 (173/176), respectively (Fig. 3C) (Fig. 5E). More than 95% of cases were correctly classified within cases with tumor cell content ≥ 0.69 (Fig. 5F).
cell content (Fig. 5C). Thus, what limited the clinical applicability of the assay is that the predictive sensitivity decreased when the determined to be 0.72 for reliable classification. To address this problem, minimum tumor cell content was addressed using machine-learning algorithms, especially in the classification of SBCLNs. Third, it was more challenging for the multiclassification model to distinguish from one another, including nMCL, MZL, and LPL/WM. Second, multiclassification has usually been much more difficult than binary classification. To cope with this problem, we split the multiclassification model into a cascade of binary classifiers. The order of an SBCLN entity to discriminate and an optimal size of gene markers were determined according to popular statistical methods, which are detailed in the Supplementary Methods. Thus, each binary classifier was finally developed based on the random forest algorithm because one-dimensionalization in advance was not necessary, and this method performed best within multiple machine-learning methods, which are detailed in the Supplementary Methods. Therefore, 102 nonmalignant biopsies from sites where SBCLNs frequently developed were included as control samples to standardize the model. As expected, the inclusion of a large number of control samples reduced the sensitivity of the model, especially to samples with low tumor cell content; however, it increased the specificity of the model. It should be noted that the validity of our model was evaluated based on our given validation set, and the tumor cell content in each sample was ≥0.3. However, the predictive specificity of the model did not significantly decrease in samples with low tumor cell content (Fig. 5C). Thus, what limited the clinical applicability of the assay is that the predictive sensitivity decreased when the model was applied to samples with low tumor cell content. To address this problem, minimum tumor cell content was determined to be 0.72 for reliable classification, since each qualified sample has a ≥95% probability of being correctly classified. Another approach was, as one of the ancillary diagnostic methods, the accuracy of the classification could be improved if specific genetic or cytogenetic aberrations were also detected. The IGH-BCL2 translocation is a hallmark of FL and is present in 80% FL cases and even 90% in low-grade FL cases, but rarely identified (3%) in non-FL SBCLN cases.21 Similarly, MYD88 L265P and BRAF V600E mutation was specifically detected in >90% LPL/WM and HCL cases, respectively. By contrast, both mutations were present in <5% other subtypes of SBCLN.21,22 Therefore, if IGH-BCL2 translocation, MYD88 L265P mutation, and BRAF V600E mutation were also detected and the results were combined with the model, the overall predictive sensitivity could increase to 0.88, and was markedly improved in samples with low tumor cell content. However, the modified model still needs to be improved in the discrimination of the MZL cases with low tumor cell content.

We also focused on our final subset of gene markers. Constitutive CCND1 and BCL2 overexpression, which is due to chromatin translocation, is a well-known hallmark of MCL and FL, respectively. BTLA is an immune checkpoint suppressor B lymphocyte attenuator, and overexpression of BTLA in CLL/SLL, which probably leads to immune escape and involved in the pathogenesis of CLL/SLL, has been described by several studies.23,24 The neural transcription factor SOX11 has emerged as a cooperative key oncogenic factor in the pathogenesis of CMCL, whereas it is not expressed in normal B cells or virtually in any other mature B-cell neoplasms.25,26 SOX11 is even highly expressed in a minority of CMCL characterized by CCND2/CCND3 rearrangements with IGKI/IGL enhancers.27 Therefore, it has also been instrumental to specifically distinguish CMCL from other SBCLNs. Previous research has illustrated that IGFBP3 (IMP-3) overexpression is seemingly restricted to several
epithelial malignancies correlated with aggressive behavior and lymphomas originating from physiologic germinal center B cells. Hodgkin lymphoma, Burkitt lymphoma, FL, and diffuse large B-cell lymphoma all demonstrated IMP-3 positivity in >80% of cases. Consistent with these findings, our data also showed that IMP-3 was a well-performing biomarker in distinguishing FL from other SBCLN cases. Moreover, some genes encoding clusters of differentiation antigens were also selected in the refined subset, including MME (CD10), FCER2 (CD23), and MS4A1 (CD20). However, most differentially expressed genes were not reported by previous studies, and further research is necessary since the association between gene overexpression and oncogenesis has not been elucidated.

There are some limitations of the model that need to be improved. First, more highly purified SBCLN cases should be included in further studies, especially cases of entities with limited sample sizes or those not included in our training cohort. Second, a larger panel of genes need to be further evaluated if the challenge lies in discriminating samples with low tumor cell content because it is usually inconvenient to enrich tumor cells in routine practice, especially for samples with only FFPE tissue available. Third, more cases with atypical/inconclusive genetic findings or belong to rare subtypes should also be included in the validation cohort, such as CLL carrying the IGH-BCL2 translocation, FL without the IGH-BCL2 translocation, Cyclin D1-negative cMCL, or SBCLN, not otherwise specified (NOS) cases. Regarding experimental methodology, consistency among different tissue types, serial dilution experiments, and intra- and interlaboratory reproducibility of the model should be determined. These works can improve the accuracy and extend the clinical applicability of the model.

In conclusion, we described a feasible model based on a digital gene expression platform that can classify SBCLNs independent of sample type with a good performance. Despite some limitations, our work provides a novel alternative for the routine diagnosis and subclassification of SBCLNs.
AVAILABILITY OF DATA AND MATERIALS
The NanoString profiling of the training cohort and the validation cohort has been deposited in the Gene Expression Omnibus (GEO) and is accessible through GEO Series accession number GSE183030.

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AUTHOR CONTRIBUTIONS
W.Z., M.X., and J.Z. designed the study, collected and analyzed data, performed statistical analysis, wrote and reviewed the paper; Q.A., D.K., and K.H.Y. reviewed pathological results. Q.A., D.K., and Z.Z provided samples; W.Z. and Z.Z. investigated GEO datasets and selected differentially expressed genes; K.S., M.Z., and Y.W. conducted flow cytometry, sequencing, and FISH studies; M.M.L. analyzed data and review the paper; Y.G. and J.W. performed digital gene expression analysis; L.Y. and H.C. review the paper.

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COMPETING INTERESTS
The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
The study was conducted in strict accordance with the guidelines formulated by the Tongji Hospital Ethics Committee (IRB ID: TJ-S1203), Wuhan, China. Written informed consent was obtained from each recruited subject in strict accordance with the Declaration of Helsinki.

ADDITIONAL INFORMATION
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