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Original Article

Tissue-Matched IgH Gene Rearrangement of Circulating Tumor DNA Shows Significant Value in Predicting the Progression of Diffuse Large B Cell Lymphoma

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Abstract

Background: Evidence has demonstrated that monitoring of the variable, diversity, and joining gene segments (VDJ) rearrangement of the immunoglobulin (Ig) genes in the circulating tumor DNA (ctDNA) is of value in predicting the outcomes of diffuse large B cell lymphoma (DLBCL). In this study, we investigated the role of VDJ rearrangement proportion in ctDNA for predicting DLBCL progression.

Methods: Patients diagnosed with newly diagnosed DLBCL were included in this study. The VDJ sequences of IgH were detected using next-generation sequencing (NGS) in formalin-fixed paraffin-embedded tissue and/or peripheral blood. The clonotype of the highest proportion in the peripheral blood was defined as the "dominant circulating clonotype," whilst the clonotype of the highest proportion in matched tissue that is detected in peripheral blood was defined as the "dominant tissue-matched clonotype." The decision tree, a machine learning-based methodology, was used to establish a progression-predicting model through a combination of "dominant tissue-matched clonotype" proportion or "dominant circulating clonotype" proportion, and the clinicopathological information, including age, sex, cell of origin, stage, international prognostic index, lactate dehydrogenase, number of extranodal involvements and β2-microglobulin.

Results: A total of 55 patients with eligible sequencing data were used for prognosis analysis, among which 36 patients had matched tissue samples. The concordance rate of "dominant circulating clonotype" and "dominant tissue-matched clonotype" was 19.44% (7/36). The decision tree model showed that the combination of extranodal involvement event and "dominant circulating clonotype" proportion (≥37%) had a clinical value in predicting the prognosis of DLBCL following combined chemotherapy (sensitivity, 0.63; specificity, 0.81; positive prediction value (PPV), 0.59; negative prediction value, 0.83; kappa value, 0.42). Noticeably, the combination of the "dominant tissue-matched clonotype" and extranodal involvement event showed a higher value in predicting the progression (sensitivity, 0.85; specificity, 0.78; PPV, 0.69; kappa value, 0.64).

Conclusion: IgH proportion detected in the ctDNA samples traced from tissue samples has a high clinical value in predicting the progression of DLBCL.

Key words: IgH gene rearrangement; circulating tumor DNA; diffuse large B cell lymphoma; progression.

Implications for Practice

The concordance rate of the highest clone detected in tissue and blood samples in patients newly diagnosed with diffuse large B cell lymphoma was low (19.44%, 7/36). Combination of "dominant tissue-matched clonotype" proportion at diagnosis and extranodal involvement event showed high values in predicting the progression of patients with newly diagnosed DLBCL (sensitivity, 0.85; specificity, 0.78; positive prediction value, 0.69; kappa value, 0.64).

Introduction

Diffuse large B cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma, with high heterogeneity in genetic characteristics, clinical manifestations, response to therapy, and prognosis.[1] Approximately 60%-70% of patients with DLBCL can be cured by the first-line treatment regimen of R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone).[2,3] Still, nearly 40% of the patients suffer from refractory or relapsed lymphoma.[4] Therefore, accurate prediction of prognosis of

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patient with DLBCL may help improve their long-term survival. The international prognostic index (IPI), the revised IPI, and the national comprehensive cancer network IPI have been identified as potential indicators of the prognosis of DLBCL. However, all 3 clinical risk scores fail to predict patients with long-term survival below 50%.[5] In addition, the fluorodeoxyglucose-positron emission tomography (PET)/computed tomography (CT), a widely used method for curative effect evaluation, has limited value in predicting the outcome of DLBCL due to its significant imprecision and potential health risk.[6-9] Thus, exploring new methods to predict the prognosis of DLBCL is needed.

Circulating tumor DNA (ctDNA) has high value in predicting treatment response and prognosis of DLBCL.[10] Recombination of variable, diversity, and joining gene segments (VDJ) of the immunoglobulin (Ig) genes is a unique marker of B-cell malignancies,[11,12] which can be detected in the plasma ctDNA samples and is emerged as a promising circulating marker for the treatment prediction of LBCL.[13,14] For example, Kurtz et al[15] found that plasma VDJ rearrangement usually preceded PET/CT detection of relapse with higher specificity and similar sensitivity in patients with DLBCL. Roschewski et al[16] quantitated the serum ctDNA concentration based on the VDJ rearrangements and found that patients with detectable ctDNA had a hazard ratio of 228 times for developing clinical progression as compared with that in patients without detectable ctDNA in DLBCL. Also, Frank et al[14] monitored lymphoma-specific VDJ clonotype ctDNA sequences and found that ctDNA assessments could risk stratify and predict the outcomes of patients with DLBCL who received chimeric antigen receptor T-cell therapy (axicabtagene ciloleucel). All of the studies emphasize the potential role of VDJ-based ctDNA monitoring in predicting the outcomes of patients with DLBCL. However, more is needed, mainly since no study has been carried out to explore the role of VDJ rearrangement proportion in predicting the progression of DLBCL in the Chinese population.

In this study, we aimed to explore the clinical value of VDJ rearrangement proportion in predicting the prognosis of patients with DLBCL. The clonotype of the highest proportion in the peripheral blood was defined as the "dominant circulating clonotype," whilst the clonotype of the highest proportion in matched tissue that is detected in peripheral blood was defined as the "dominant tissue-matched clonotype." The "dominant tissue-matched clonotype" proportion or "dominant circulating clonotype" proportion and the clinicopathological information, including age, sex, cell of origin(COO), stage, IPI, lactate dehydrogenase (LDH), number of extranodal involvements, and β2-microglobulin were included into a progression model to predict the prognosis of patients with DLBCL. This model may provide an effective tool for clinicians to evaluate the prognosis of DLBCL, thereafter helping improve the outcome of Chinese patients with DLBCL.

Methods

Patients and Sample Collection

A total of 60 patients with DLBCL were enrolled at Union Hospital, Tongji Medical College, Huazhong University of Science and Technology from July 2017 to December 2020. Eligible patients were ≥18 years old; newly diagnosed with DLBCL with no treatment previously. The patients were followed up to November 2021. Peripheral blood samples were collected from all the patients and preserved in EDTA-containing cell-free DNA collection tubes. Tumor samples were collected from 37 patients and fixed by formalin, then embedded in paraffin. The key endpoint was to assess the ability of VDJ rearrangement proportion in ctDNA at diagnosis to predict the progression-free survival (PFS) of patients with DLBCL. The patients received R-CHOP (rituximab 375 mg/m^2 for day 1, cyclophosphamide 750 mg/m^2 for day 2, adriamycin 50 mg/m^2 for day 2, vincristine 2 mg for day 2, and prednisone 100 mg for days 2-6) regimen. A PFS event was defined as disease relapse or death during the follow-up period. This study was approved by the Ethics Committees of Cancer Center, Union Hospital, Tongji Medical College, and Huazhong University of Science and Technology. The need for informed consent was waived because only deidentified patient data were used.

Identification of the Clonotypes of IgH by Next-Generation Sequencing

Genomic DNA (gDNA) was isolated from archived formalin-fixed, paraffin-embedded (FFPE) tissue blocks using the Maxwell RSC DNA FFPE kit (Promega, Madison, WI, USA) referring to the manufacturer's descriptions. ctDNA was isolated from peripheral blood samples using CWhipro Circulating DNA Midi Kit (CWBIO, Jiangsu, China) with a spin column-based cfDNA extraction technique. Briefly, after adding the binding buffer to the proteinase-digested plasma sample (plasma volume varied from 2 to 3.5 mL), the plasma sample was filtered through the spin column under vacuum pressure (CWBIO). To minimize the white blood cells contamination, we performed 2-step centrifugation for the plasma sample, 550 g for 10 minutes and 12519 g for 10 minutes. Then, the magnetic bead purification was used in the sequencing library preparation for IgH VDJ recombination detection (Shanghai Yuanqi Biomedical Technology Co., Ltd., Shanghai, China). First, the multiplex PCR was performed to amplify the VDJ-rearranged CDR3 region of IgH gene; then 1.8 volume of Agencourt AMPure XP magnetic beads was added to the multiplex PCR product to enrich fragments above 150 bp. Following that, the second PCR was performed using primers containing sequencing adaptors and index; and then 1 volume of Agencourt AMPure XP magnetic beads was added to the PCR product to enrich fragments above 250 bp. Finally, DNA fragments above 250 bp were captured and sequenced on the Illumina MiSeq platform.

Quality control of fastq data was performed by FASTP software, and sequences were then spliced, removed redundancy, and counted by VSEARCH software. The alignment of sequences was performed by IGBLASTN software. Finally, consensus sequences of the same VDJ classification were counted as one clone, and the corresponding ratio was calculated based on the proportion in the total number of sequences. The proportion of each clonotype in a sample was determined by calculating the number of sequencing reads for each clonotype divided by the total number of passed sequencing reads in the sample, and the clonotypes in tissue samples or blood samples without matched tissues with >5% proportion were retained.[17] The clonotype in the ctDNA samples which ranked the highest proportion in the matched tissue samples was defined as "dominant tissue-matched clonotype," while the highest proportion clonotype in the blood

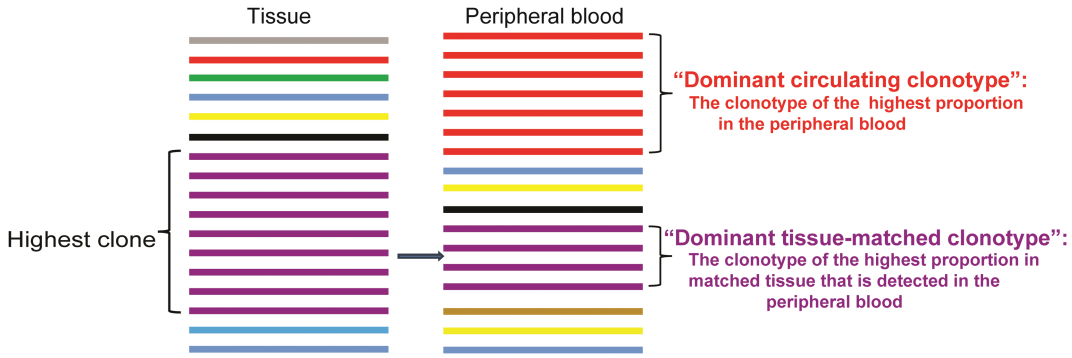

Figure 1. Illustration for the concepts of "dominant circulating clonotype" and "dominant tissue-matched clonotype." The clonotype of the highest proportion in the peripheral blood was defined as the "dominant circulating clonotype," whilst the clonotype of the highest proportion in matched tissue that is detected in the peripheral blood was defined as the "dominant tissue-matched clonotype."

ctDNA samples was defined as "dominant circulating clonotype," as shown in Figure 1.

Statistical Analysis

The decision tree, a machine learning-based methodology, was used to assess the significant clinical predictors of survival rate.[18] First, the random forest algorithm was used to build a random forest of a fixed number of classification trees according to the explanatory variables, including the "dominant tissue-matched clonotype" proportion or "dominant circulating clonotype" proportion, age, IPI, and Ann Arbor stage. The dependent variable was PFS (no or yes). Then, the importance of each explanatory variable was assessed. Variables associated with a mean decrease in accuracy >1% were then included in the classification tree construction. After the most important explanatory variables were identified, the rpart algorithm was used to decide which of these variables to split and which splitting value to take at each step of the tree's construction. To cope with the overfitting and instability inherent in the decision tree, a 10-fold cross-validation procedure was applied. The area under the receiver operating characteristic curve (ROC) was used to assess overall model discrimination. Univariate Cox proportional hazards regression models were used to assess the effect of candidate variables on the prediction of progression. Chi-square tests and Fisher's exact tests were used to compare the differences between groups. Kaplan-Meier curves with log-rank tests were used to assess the relationship between age, number of extranodal involvement, LDH level, COO and stage, and the PFS of patients with primary DLBCL. R software, version 3.4.3 (Chicago, IL), and GraphPad (6.0) were used for statistical analyses. A 2-sided P value < .05 was considered as a difference with significance.

Results

Patient Characteristics and the Influencing Factor of Prognosis

A total of 55 patients were included in the following analysis with complete clinical information and/or qualified sequencing data. Among the 55 patients, 52 had the blood IgH rearrangement data, among which 36 had the matched tissues IgH rearrangement data. Twenty-five (45.45%) out of the 55 cases were male; 22 cases (40.00%) were diagnosed at the age of ≤60 years; 37 cases (67.27%) were non-GCB type; 26 cases (47.27%) were at stage I-II, as shown in Table 1. The patients were followed until November 2021, and 7 patients (12.73%) died during the follow-up period (median of 23.40 months, ranged from 0.27 to 52.43 months).

Table 1. Clinicopathological characteristics of the 55 patients with DLBCL.

Clinical information	*n* (%)
Sex	
Male	25 (45.45)
Female	30 (54.55)
Age (years)	
≤60	22 (40.00)
>60	33 (60.00)
COO	
GCB	17 (30.91)
Non-GCB	37 (67.27)
NA	1 (1.82)
Stage	
I-II	26 (47.27)
III-IV	29 (52.73)
Number of extranodal involvement	
0-1	37 (67.27)
≥2	17 (30.91)
NA	1 (1.82)
LDH	
Normal	26 (47.27)
High	29 (52.73)
β2-microglobulin	
Normal	38 (69.09)
High	16 (29.09)
NA	1 (1.82)
IPI	
0-1	17 (30.91)
2	18 (32.73)
3	8 (14.55)
4-5	10 (18.18)
NA	2 (3.64)

Abbreviations: NA, not available; COO, cell of origin; LDH, lactate dehydrogenase; IPI, international prognostic index.

The Kaplan-Meier curves with log-rank test showed that the advanced stage (III-IV) was a significant influencing factor of patients' PFS (Fig. 2A, $P = .033$). In contrast, age (Fig. 2B), COO Hans classification (Fig. 2C), the number of extranodal involvement (Fig. 2D), and LDH level (Fig. 2E) showed no significant effect.

Clinical Value of Blood "Dominant Circulating Clonotype" in Predicting the Progression of DLBCL Patients

To assess the clinical value of blood IgH rearrangement in predicting the progression of patients with DLBCL, we first assessed the effect of blood "dominant circulating clonotype" on the progression prediction in 52 patients with DLBCL. The decision tree model with the proportion of blood "dominant circulating clonotype" and clinical features including age (>60), sex, COO (GCB, non-GCB), stage (I, II, III, and IV), IPI (0-5), LDH (high, normal), number of extranodal involvements, and β2-microglobulin (high, normal) to predict the progression of DLBCL cases. The optimal model demonstrated that patients with ≥1 extranodal involvement site and ≥37% proportion of "dominant circulating clonotype" were predicted to progress after treatment (Fig. 3A), with an AUC (area under the curve) of 0.724, kappa value of 0.42, sensitivity of 0.63, specificity of 0.81, positive prediction value (PPV) of 0.59 and negative prediction value (NPV) of 0.83 (Fig. 3B). Also, the PFS of the progression group predicted by the model was significantly longer than the non-progression group (Fig. 3C). The results showed that a combination of extranodal involvement number and blood "dominant circulating clonotype" showed a specific clinical value in predicting the progression of DLBCL.

Clinical Value of Blood "Dominant Tissue-Matched Clonotype" in Predicting the Progression of Patients With DLBCL

Also, we assessed the effect of "dominant tissue-matched clonotype" on the progression of DLBCL in 36 patients. Only 7 patients presented the "dominant circulating clonotype" consistent with the "dominant tissue-matched clonotype" (concordance rate of 19.44%). Also, we constructed the decision tree model with the proportion of blood "dominant

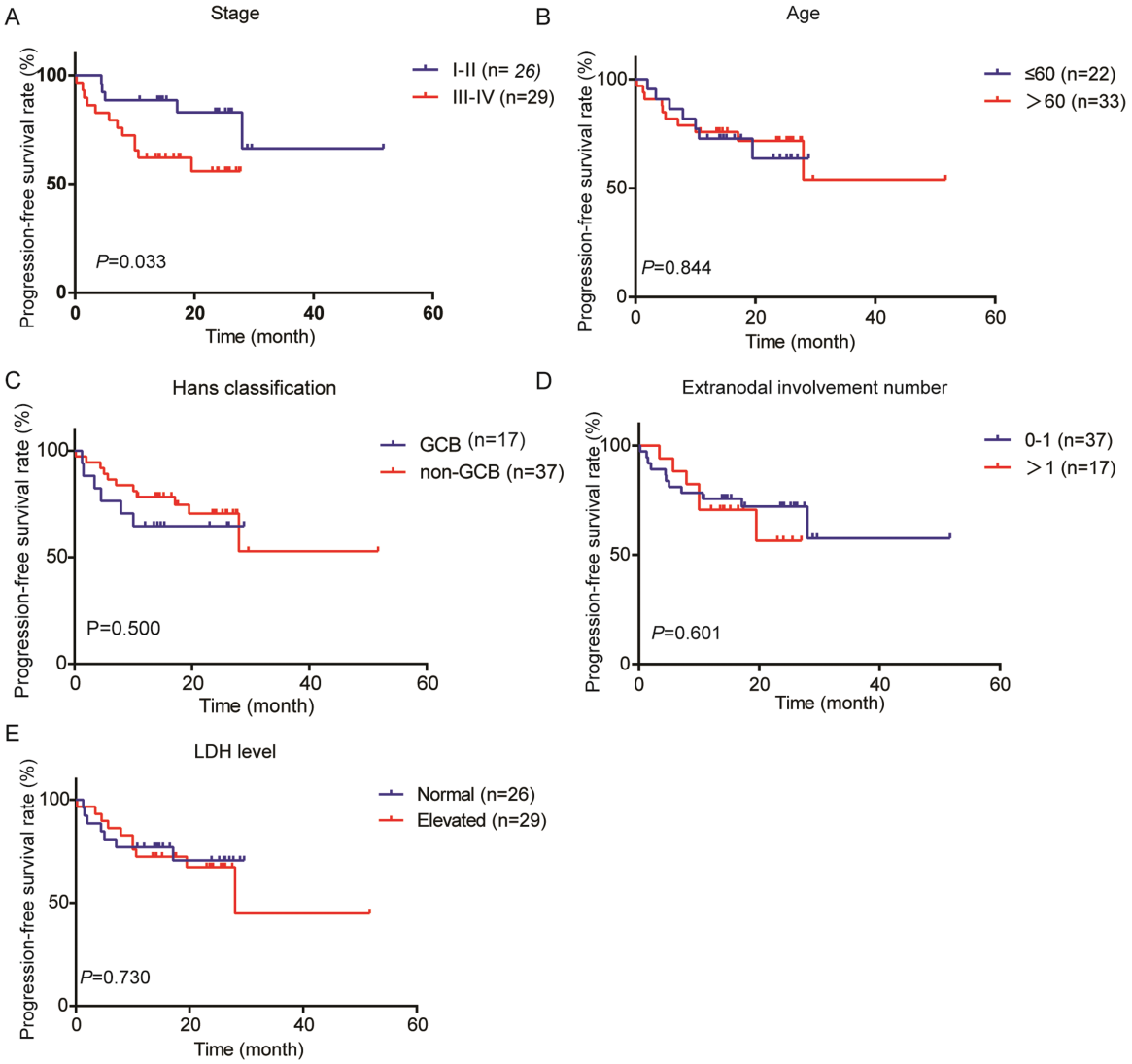

Figure 2. The relationship between clinical features and the PFS of patients with DLBCL. Kaplan-Meier curves with log-rank tests were applied to assess the (**A**) stage, (**B**) age, (**C**) COO Hans classification, (**D**) extranodal involvement number, and (**E**) LDH level.

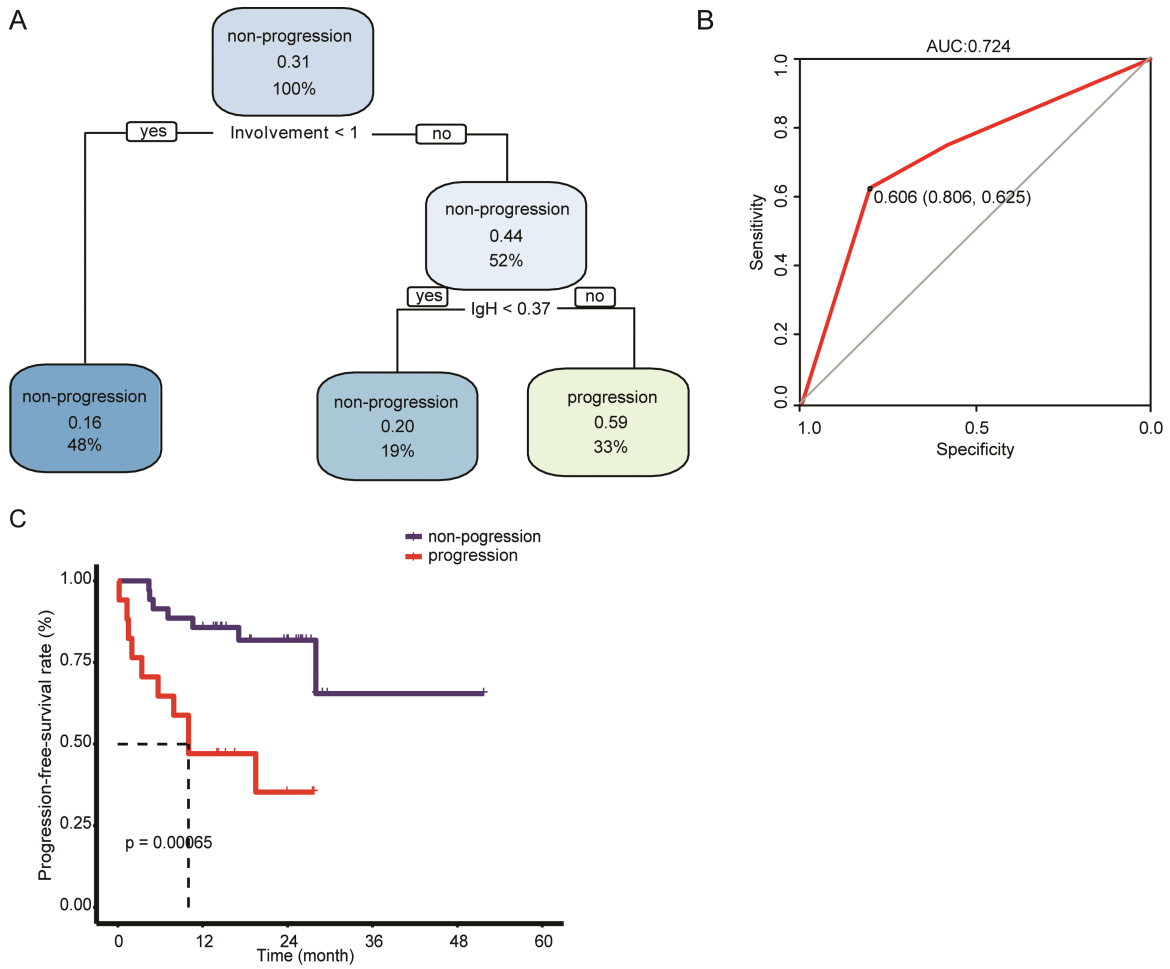

Figure 3. Clinical value of blood "dominant circulating clonotype" in predicting the progression of patients with DLBCL. (**A**) The decision tree model constructed to predict the progression of patients with DLBCL (The number in the second row of the branch represents the proportion of patients who progressed in that branch. The percentage of the third row in the branch represents the percentage of the number of patients in the branch in the total number of patients included in the decision tree). (**B**) ROC analysis of the sensitivity, specificity, kappa value of the decision tree model. (**C**) Kaplan-Meier curve showed the PFS of the progression and non-progression groups predicted by the model. Second row: "Index clone" should be replaced by "IgH"

tissue-matched clonotype" identified from tissues and clinical features including age (>60), sex, COO (GCB, non-GCB), stage (I, II, III, and IV), IPI (0-5), LDH (high, normal), number of extranodal involvements, and β2-microglobulin (high, normal) to predict the progression of DLBCL cases. The optimal model demonstrated that patients with ≥80% proportion of "dominant tissue-matched clonotype" in ctDNA samples or patients with a "dominant tissue-matched clonotype" proportion between 47% and 80% together with extranodal involvement were predicted to be progressors after treatment (Fig. 4A), with an AUC of 0.85, kappa value of 0.64, sensitivity of 0.85, specificity of 0.78, PPV of 0.69, and NPV of 0.90 (Fig. 4B). Also, the PFS of the progression group predicted by the model was significantly longer than the non-progression group (Fig. 4C). Moreover, the 36 patients were divided into 2 groups, the "0" group without detectable blood "dominant tissue-matched clonotype" and the ">0" group with detectable blood "dominant tissue-matched clonotype." The clinicopathologic features of the 2 groups were compared, and the results showed that patients with high β2-microglobulin level tended to have detectable "dominant tissue-matched clonotype" (Table 2). These results showed that the proportion of "dominant tissue-matched clonotype" showed a higher value in predicting the prognosis of DLBCL as compared with the proportion of blood "dominant circulating clonotype" without tissue confirmation.

Discussion

Complete eradication of tumor clones is required to cure DLBCL, or it is inevitable to relapse.[19] The VDJ sequences of Ig genes are attractive targets as they are virtually 100% tumor-specific markers of B-cell lymphomas that remain stable throughout the disease course.[20] In response to antigen stimulation, each B-cell can form a single productive IgH VDJ sequence by joining a VH (variable), a D (diversity), and a JH (joining) segment together from a large pool of these segments. The sequence is unique and can be inherited in all the progeny of this B-cell, therefore tagging individual mature B-cell and its offspring. Thus, a dominant clonotype is usually found in malignant B cell lymphomas owing to the over-proliferation of one B cell.[21] The proportion of dominant tissue-matched clonotype detected in the blood sample indirectly reflects the tumor burden of patients. Thus, the higher proportion of the index clone detected in the blood

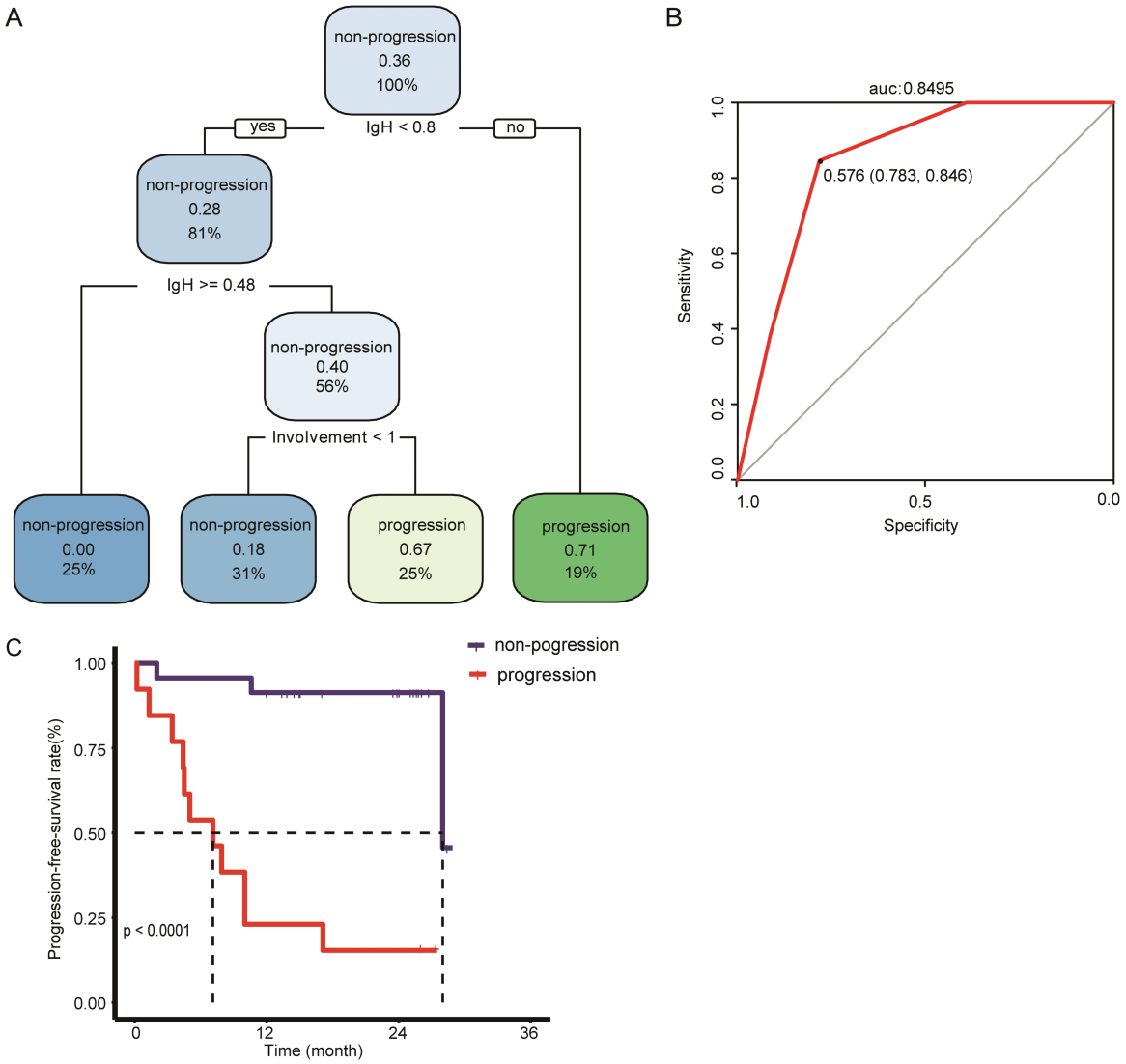

Figure 4. Clinical value of blood "dominant tissue-matched clonotype" in predicting the progression of patients with DLBCL. (**A**) The decision tree model constructed to predict the progression of patients with DLBCL (The number in the second row of the branch represents the proportion of patients who progressed in that branch. The percentage of the third row in the branch represents the percentage of the number of patients in the branch in the total number of patients included in the decision tree). (**B**) ROC analysis of the sensitivity, specific, kappa value of the decision tree model. (**C**) Kaplan-Meier curve showed the PFS of the progression and non-progression groups predicted by the model.

is associated with a worse prognosis. Recently, studies have demonstrated that high-throughput sequencing of Ig genes (Ig-HTS) from blood ctDNA has been used as a disease biomarker for B-cell malignancies, including DLBCL.[15,16,22,23] Noticeably, ctDNA quantification based on IgH sequencing showed high values in predicting the curative effects in patients with DLBCL.[14,16] Here, we explored the clinical value of the "dominant tissue-matched clonotype" proportion in predicting the progression of patients with DLBCL in the Chinese population. The results illustrated that a higher proportion of detected "dominant tissue-matched clonotype" predicted a higher risk of progression in patients with DLBCL.

VDJ clonotypes detected by next-generation sequencing (NGS) have distinct advantages including identification of the V, D, and J segments, clonal relationship between histologically distinct lymphomas, somatic hypermutation in the V region, and B-cell receptor stereotypy based on HCDR3 length and composition, as well as monitoring minimal residual disease (MRD) with high sensitivity.[24–26] NGS-based VDJ rearrangement detection has been gradually applied to the diagnosis and prognosis assessment of lymphomas. Sarkozy et al[27] found that the high level of ctDNA based on the VDJ rearrangement was an independent factor affecting PFS in patients with follicular lymphoma. Also, the detection of ctDNA encoding clonal rearranged VDJ sequences showed higher analytical sensitivity and enhanced tumor specificity compared to imaging scans in DLBCL.[28] Roschewski et al[16] retrospectively analyzed the clinical role of ctDNA encoding VDJ sequences in predicting the progression of DLBCL with combination chemotherapy. The results revealed that the ctDNA could be detectable in 15 (88%) of 17 patients with DLBCL who experienced disease relapse after complete remission prior to disease relapse, highlighting the critical role of IgH-based ctDNA detection in predicting the progression of DLBCL. Here, we assessed the clinical value of IgH proportion detected in the ctDNA samples using the decision tree. According to the IgH proportion of "dominant

Table 2. Clinicopathological characteristics of the 36 patients with DLBCL.

Clinical information	Proportion of "dominant tissue-matched clonotype"		*P* value
	0 (*n*, %)	>0 (*n*, %)	
Sex			
Male	11 (42.31)	6 (60.00)	.463
Female	15 (57.69)	4 (40.0)	
Age (years)			
≤60	6 (23.08)	5 (50.00)	.232
>60	18 (69.23)	5 (50.00)	
COO			
GCB	12 (46.15)	1 (10.00)	.070
Non-GCB	13 (50.00)	9 (90.00)	
NA	1 (3.85)	0 (0.00)	
Stage			
I-II	15 (57.69)	3 (30.00)	.264
III-IV	11 (42.31)	7 (70.00)	
Number of extranodal involvement			
0-1	20 (76.92)	6 (60.00)	.413
≥2	6 (23.08)	4 (40.00)	
LDH			
Normal	13 (50.00)	4 (40.00)	.717
High	13 (50.00)	6 (60.00)	
β2-microglobulin			
Normal	24 (92.31)	6 (60.00)	.039
High	2 (7.69)	4 (40.00)	
IPI			
0-1	8 (30.77)	3 (30.00)	.925
2	9 (34.62)	3 (30.00)	
3	3 (11.54)	1 (10.00)	
4-5	4 (15.38)	3 (30.00)	
NA	2 (7.69)	0 (0.00)	

Abbreviations: NA, not available; COO, cell-of-origin; LDH, lactate dehydrogenase; IPI, international prognostic index.

circulating clonotype" detected in ctDNA sample without tissue identification, the decision tree showed that the combination of extranodal involvement event and blood "dominant circulating clonotype" (≥37%) showed some clinical value in predicting the prognosis of DLBCL (sensitivity, 0.63; specificity, 0.81; PPV, 0.59; NPV, 0.83; kappa value, 0.42). Due to lacking tissue trace, the IgH rearrangement detected in blood samples may not be convincible as "multiple dominant" caused by VDJ-recombination errors, failure of allelic exclusion, intraclonal diversification, and "true" bi- or oligoclonality.[29] However, we did find the "dominant circulating clonotype" shows a value in predicting the progression of DLBCL, suggesting that it is reflective of tumor burden to some degree.

Also, we assessed the clinical value of IgH proportion detected in the ctDNA samples traced from tissue "dominant tissue-matched clonotype." The decision tree showed that the proportion of "dominant tissue-matched clonotype" has a higher predictive value as compared with that of "dominant circulating clonotype" in predicting the PFS (sensitivity, 0.85; specificity, 0.78; PPV, 0.69; kappa value, 0.64), emphasizing the importance of tissue confirmation at diagnosis. Nevertheless, the sample size of this series was too small. This hypothesis needs to be further evaluated in a larger cohort with an adequate exploration of the clinical value, such as its prediction ability of curative effect and prognosis, as well as the MRD monitoring, of IgH proportion in blood samples with tissue confirmation.

Last, we should claim the limitations of the current study. One is that we showed that detection of the IgH rearrangement only in the blood samples without tissue samples shows little significance to the clinic, thus the tumor clone sequence must be detected in the primary tumor samples to ensure the sequences that may be traced in the following blood samples. In addition, leukocyte gDNA contaminants could not be ruled out of this study. Thus, the proportion of index clone detected in the blood sample can only indirectly reflect the tumor burden of patients. This is a limitation of this study. To minimize the gDNA contamination, we performed 2-step centrifugation for the plasma sample, 550 g for 10 minutes and 12519 g for 10 minutes. In addition, if aberrantly high concentrations of cfDNA were detected, specifically higher than 2.0 ng/μL, such samples were excluded from this assay. Advanced technology should be developed for further application.

Collectively, this study demonstrated that the IgH proportion detected in the ctDNA samples traced from tissue

samples showed a high clinical value in predicting the progression of DLBCL.

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Conflict of Interest

Zhonghe Ke is an employee of Shanghai Rightongene Biotechnology Ltd. The other authors indicated no financial relationships.

Author Contributions

Conception/design: Q.L., L.Z. Provision of study material or patients: X.L., T.L., F.Z., Q.W., Q.L., L.Z. Collection and/or assembly of data: K.Z., X.Z., X.L., T.L., Z.K., Q.W., Q.L. Data analysis and interpretation: Z.K., B.X., L.Z. Manuscript writing: K.Z., X.Z., B.X., Q.L., L.Z. Final approval of manuscript: All authors.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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