Pharmacogenomics and Personalized Medicine

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ORIGINAL RESEARCH

CHN1 is a Novel Prognostic Marker for Diffuse Large B-Cell Lymphoma

This article was published in the following Dove Press journal:
Pharmacogenomics and Personalized Medicine

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Purpose: Diffuse large B-cell lymphoma (DLBCL) is the most common B-cell malignancy. Thirty to forty percent of DLBCL patients still experience relapse or develop refractory disease even with standard immunochemotherapy, leading to a poor prognosis. Currently, although several gene-based classification methods can be used to predict the prognosis of DLBCL, some patients are still unable to be classified. This study was performed to identify a novel prognostic biomarker for DLBCL.

Patients and Methods: A total of 1850 B-cell non-Hodgkin lymphoma (B-NHL) patients in 8 independent datasets with microarray gene expression profiles were retrieved from the Gene Expression Omnibus (GEO) database and Lymphoma/Leukemia Molecular Profiling Project (LLMPP). The candidate genes were selected through three filters in a strict pipeline. Survival analysis was performed in two independent datasets of patients with both gene expression data and clinical information. Gene set enrichment analysis (GSEA) and the CIBERSORT algorithm were used to explore the biological functions of the genes.

Results: We identified 6 candidate genes associated with the clinical outcome of DLBCL patients: *CHN1, CD3D, CLU, ICOS, KLRB1* and *LAT*. Unlike the other five genes, *CHN1* has not been previously reported to be implicated in lymphoma. We also observed that *CHN1* had prognostic significance in important clinical subgroups; in particular, high *CHN1* expression was significantly related to good outcomes in DLBCL patients with the germinal center B-cell-like (GCB) subtype, stage III–IV, or an International Prognostic Index (IPI) score > 2. Multivariate Cox regression analysis of the two datasets showed that *CHN1* was an independent prognostic factor for DLBCL. Additionally, GSEA and CIBERSORT indicated that *CHN1* was correlated with cell adhesion and T cell immune infiltration.

Conclusion: Our data indicate for the first time that high *CHN1* expression is associated with favorable outcomes in DLBCL patients, suggesting its potential utility as a prognostic marker in DLBCL.

Keywords: *CHN1*, diffuse large B-cell lymphoma, prognosis, biomarker

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common B-cell malignancy and accounts for 30%–40% of all non-Hodgkin's lymphomas (NHLs).[1] Although R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) improves the outcome of DLBCL, 30%–40% of patients will eventually relapse and develop refractory disease, with inferior prognoses.[2–4] Therefore, an accurate evaluation of prognosis in advance is important to guide appropriate treatment.

The International Prognostic Index (IPI) is a popular tool for predicting survival in patients with DLBCL and includes five clinical indicators: age, Ann Arbor stage, serum

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http://doi.org/10.2147/PGPM.S301718

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lactate dehydrogenase, performance status, and a number of extranodal disease sites.[5] However, the IPI cannot be used to accurately predict the clinical outcome of many patients[6,7] or reflect the molecular heterogeneity of DLBCL.

Recently, researchers have attempted to explore genetic alterations and molecular heterogeneity for risk stratification and prognosis prediction in patients with DLBCL. Alizadeh et al first divided DLBCL into germinal center B-cell-like (GCB) and activated B-cell-like (ABC) subtypes by analyzing gene expression profiles, with a small unclassified group (10–15%).[8] Patients with the ABC subtype had more unfavorable outcome than those with the GCB subtype following R-CHOP treatment, with a 5-year survival rate of 56% for patients with the ABC subtype and 78% for patients with the GCB subtype.[9] Chapuy et al discovered five subsets of DLBCL (Cluster 1–5) with different coordinated genetic signatures and prognoses, but 4% of tumors were undefined.[10] The coordinated genetic signature was an independent prognostic factor, of which C1 and C4 DLBCL had a better prognosis than C3 and C5. Wright et al developed the LymphGen algorithm to categorize DLBCL into seven genetic subtypes including MCD (*MYD88*^{L265P} and *CD79B* mutations), N1 (*NOTCH1* mutations), A53 (aneuploid with *TP53* inactivation), BN2 (*BCL6* translocations and *NOTCH2* mutations), ST2 (*SGK1* and *TET2* mutations), EZB-MYC^{+} (EZB, *EZH2* mutations and *BCL2* translocations), and EZB-MYC^{-}, and the remaining cases were classified as "other". Patients with MCD had a poor prognosis, and patients with BN2 had a favorable prognosis.[11,12] However, a proportion of patients still failed to be classified into a specific subtype according to the aforementioned classification, and it was difficult to predict prognosis in these patients. Overall, there remains a strong need to identify novel and easy-to-use prognostic markers for DLBCL.

In the present study, we comprehensively analyzed the gene expression profiles of B-cell non-Hodgkin lymphoma (B-NHL) patients from the Gene Expression Omnibus (GEO) database and Lymphoma/Leukemia Molecular Profiling Project (LLMPP). By applying three filters in a strict pipeline, we found that high *CHN1* expression was associated with good overall survival in DLBCL, and thus characterized its clinical features and significance.

Patients and Methods

Data Collection

The microarray gene expression profiles of 1850 B-NHL patients in total were retrieved from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and LLMPP (http://llmpp.nih.gov). The GSE132929 dataset, which includes tumor tissue samples of follicular lymphoma (FL, n = 65), DLBCL (n = 95) and Burkitt lymphoma (BL, n = 59), was used as the discovery cohort to identify differentially expressed genes (DEGs). Five independent datasets of DLBCL (GSE34171, n = 91; GSE25638, n = 26), FL (GSE65135, n = 14; GSE93261, n = 147), and BL (LLMPP, n = 33) were used as testing cohorts to confirm the reproducibility of the DEGs. In addition, a total of 1332 DLBCL patients were included in the survival analysis and gene set enrichment analysis (GSEA) after excluding patients with no clinical data, including 414 patients from GSE10846 and 928 patients from GSE117556. All datasets except GSE117556, which was performed on the Illumina HumanHT-12 WG-DASL V4.0 R2 expression Beadchip, were performed on the Affymetrix Human Genome U133 Plus 2.0 Array. The details of all datasets are shown in Supplementary Table S1.

Data Processing

The raw data of the Affymetrix microarray were processed into clean data by using the "affy" R package. Data processing included RMA background correction, quantile normalization, log_2 transformation, and median polishing algorithm summarization. The Series Matrix File format data of Illumina were normalized using the "lumi" R package. The data were annotated by converting the different probe IDs to gene IDs based on the platform annotation files. The batch effect of multiple microarrays in the integrated analysis was identified and removed using ComBat function in the "sva" R package. DEGs were identified using the "limma" R package.

The final candidate genes were selected by applying three filters of the strict pipeline (Figure 1). According to the ranking of aggressiveness of B-NHL, which from low to high is FL, DLBCL, and BL, the expression trends of the DEGs in the discovery cohort that were consistent or opposite to disease aggressiveness were screened out (Filter 1). The cutoff criteria for identifying DEGs were $|log_2 \text{fold change (FC)}| > 1$ and $p < 0.05$. Afterward, these DEG values were extracted in the testing cohorts to validate the reproducibility. Only the genes that displayed consistent trends in the discovery and testing cohorts were chosen for the next step (Filter 2), and the cutoff criteria were $|log_2 FC| > 0.8$ and $p < 0.05$.

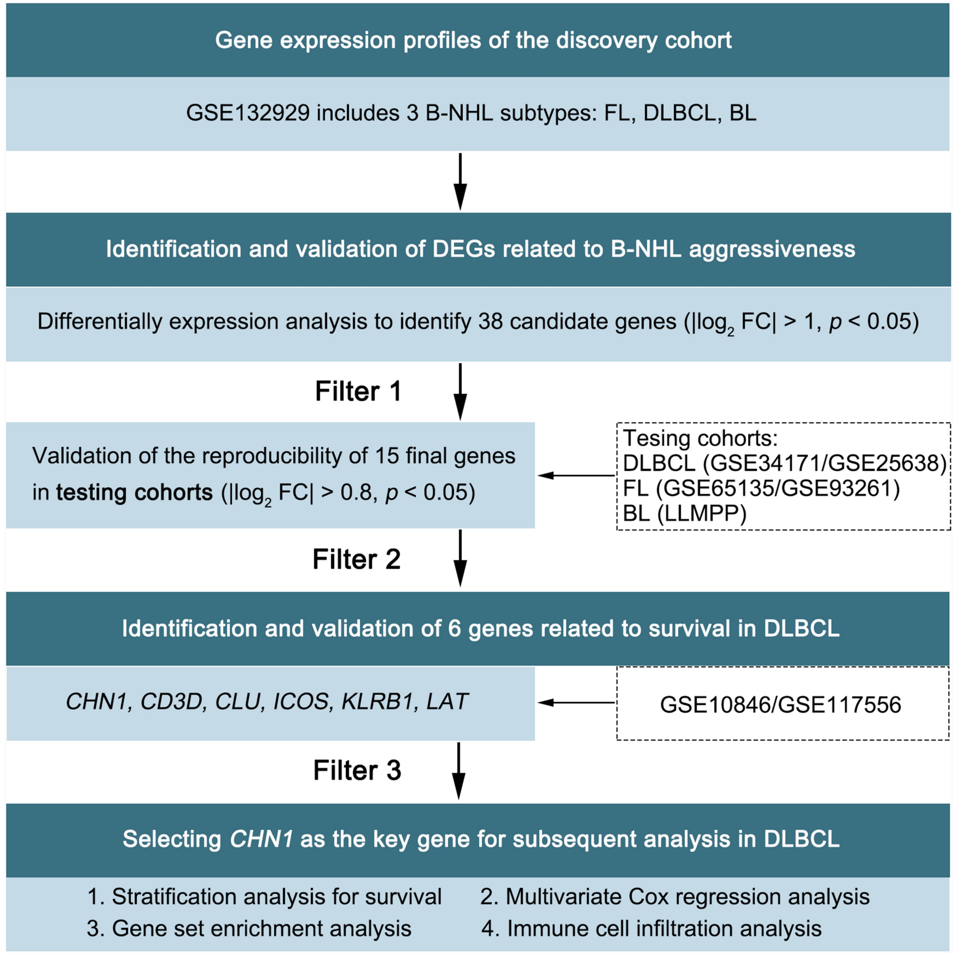

Figure 1 Study workflow. DEGs, differentially expressed genes; FC, fold change.

Survival Analysis

Survival analysis was conducted to test the ability of the genes to predict the prognosis of DLBCL by using both the GSE10846 and GSE117556 datasets (Filter 3). Patients were categorized into high and low expression group based on the median value of gene expression. We utilized the "survival" R package to draw Kaplan–Meier survival curves and compared them using the Log rank test.

Gene Set Enrichment Analysis

GSEA (version 4.0.3) was used to analyze the enrichment of GO terms and KEGG pathways between the high and low *CHN1* expression groups to investigate potential biological functions and enriched pathways. The c2.cp.kegg.v7.0.symbols and c5.bp.v7.0.symbols molecular signatures were used as the reference gene sets for analysis. The nominal p-value and normalized enrichment score (NES) were calculated to depict the enriched GO terms and KEGG pathways. The cutoff criteria were nominal p-value < 5% and |NES| > 1.

Immune Cell Infiltration Analysis

CIBERSORT, a deconvolution algorithm, is utilized to calculate the proportion of immune cells in tumors based on gene expression profiles. The LM6 gene signature was set as the reference profile for immune infiltration analysis. We compared the distribution of each immune cell between the high and low *CHN1* expression groups using the Wilcoxon test.

Statistical Analysis

Multivariate Cox regression analyses were conducted to evaluate independent prognostic factors. Continuous variables were analyzed using Student's t-test or the Mann–Whitney U-test. Categorical variables were analyzed using Fisher's exact test. All statistical analyses were performed via SPSS version 25 (IBMCorp., Armonk, N.Y., USA) and

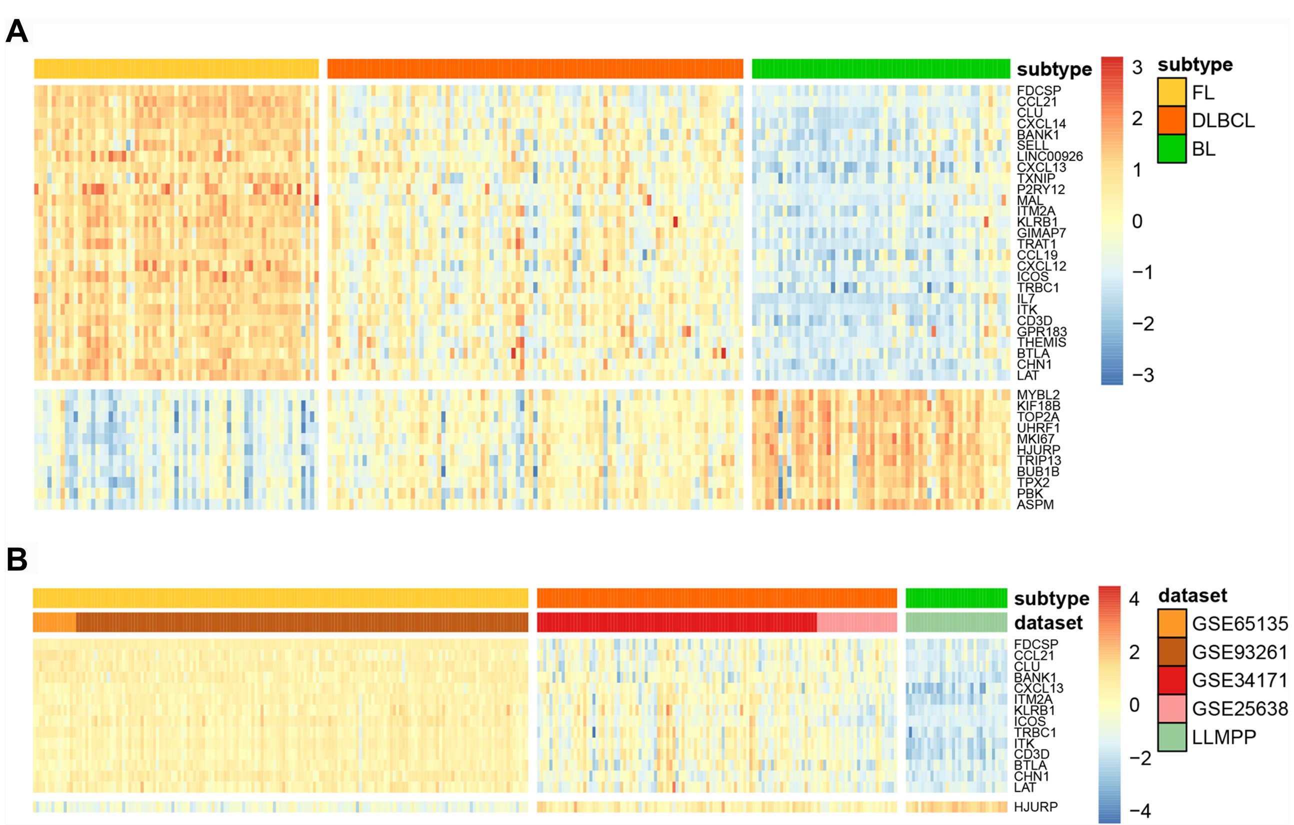

Figure 2 Identification and validation of DEGs related to lymphoma aggressiveness. (**A**) Heatmap of 38 DEGs in the discovery cohort (GSE132929). (**B**) Heatmap of 15 DEGs in the testing cohorts. Each column represents a sample, and each row represents the gene expression level. Low expression is marked in blue, and high expression is marked in red.

R software version 3.6.1 (R core Team, Vienna, Austria). A value of $p < 0.05$ was considered statistically significant.

Results

Identification and Validation of DEGs Related to B-NHL Aggressiveness

B-NHL mainly encompasses indolent FL, aggressive DLBCL, and highly aggressive BL according to the clinical course of disease. Given that aggressive lymphoma characteristics typically indicate a poor prognosis,[13–18] we analyzed the gene expression profiles of FL (n = 65), DLBCL (n = 95), and BL (n = 59) patients from the GSE132929 dataset as a discovery cohort to identify DEGs. A total of 38 DEGs were obtained (Figure 2A), and we further confirmed the reproducibility of these DEGs in the additional five testing cohorts, including 311 B-NHL patients from the GSE34171, GSE25638, GSE65135, GSE93261 and LLMPP. Only 15 of the 38 genes displayed a trend consistent with the results of the discovery cohort (Figure 2B, Supplementary Table S2), of which 14 were gradually increased in highly aggressive BL, aggressive DLBCL and indolent FL, and 1 exhibited the opposite trend.

Identification and Validation of 6 Genes Related to Survival in DLBCL Patients

Then, we conducted survival analysis to explore the prognostic value of the 15 genes in DLBCL patients. The clinical information of 414 patients from GSE10846 and 928 patients from GSE117556 are summarized in Supplementary Table S3. Six genes, namely, *CHN1, CD3D, CLU, ICOS, KLRB1* and *LAT*, were identified to be associated with overall survival (OS). Kaplan–Meier curves showed that the high expression of these genes was correlated with a prolonged survival time in both GSE10846 (Figure 3A–F, $p < 0.05$) and GSE117556 (Figure 3G–L, $p < 0.05$).

CD3D, CLU, ICOS, KLRB1 and *LAT* appear to be prognostic factors in a range of malignancies, such as B-NHL,[19,20] colorectal cancer,[21,22] ovarian cancer,[23] bladder cancer,[24] breast cancer,[25,26] cervical cancer,[27] and lung cancer,[28–31] and play important roles in lymphoma

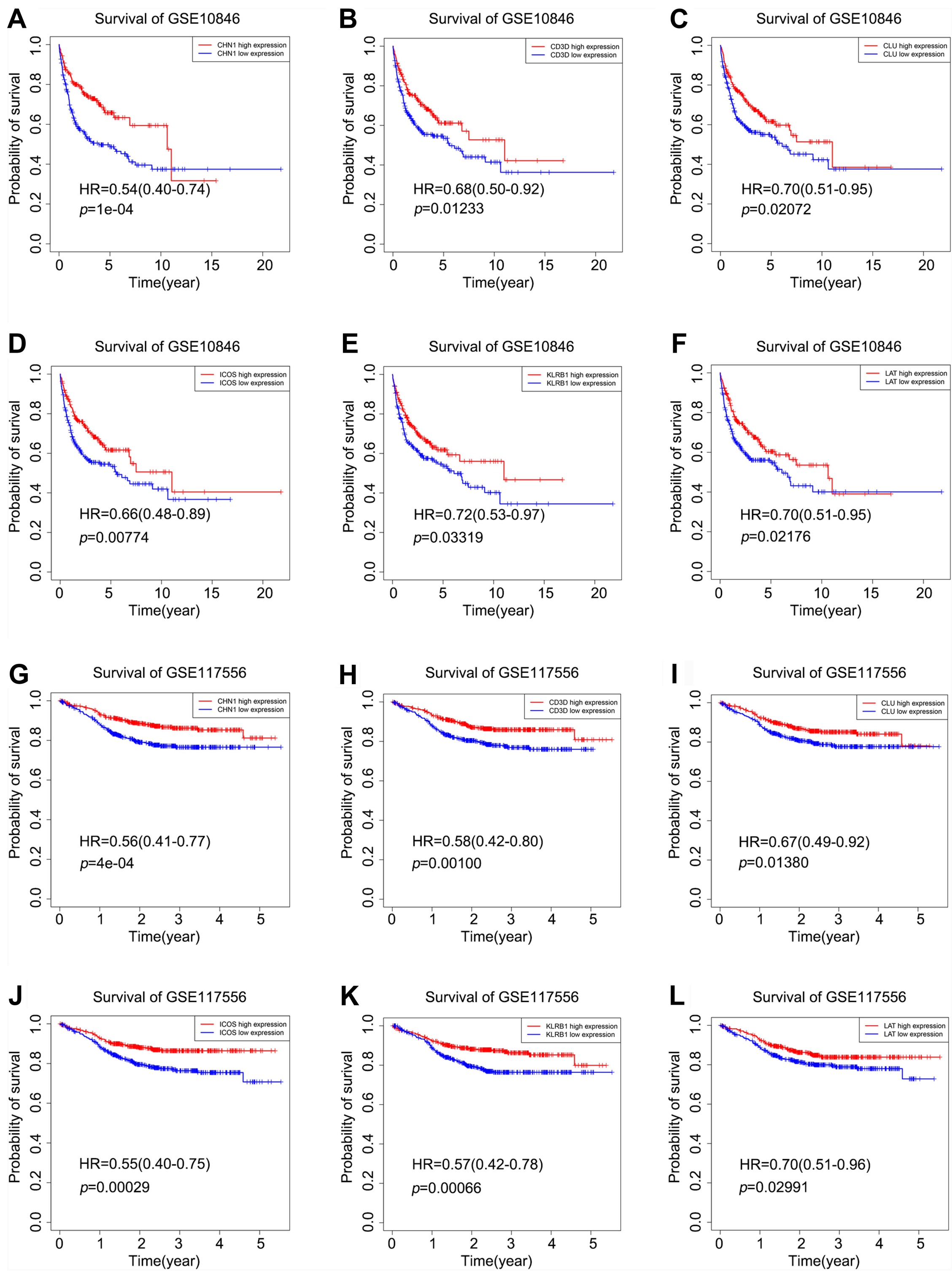

Figure 3 The prognostic significance of *CHN1, CD3D, CLU, ICOS, KLRB1* and *LAT* in DLBCL. Kaplan–Meier curves of OS between the high and low expression groups in GSE10846 and GSE117556 stratified by 6 genes: (**A**, **G**) stratified by *CHN1*, (**B**, **H**) stratified by *CD3D*, (**C**, **I**) stratified by *CLU*, (**D**, **J**) stratified by *ICOS*, (**E**, **K**) stratified by *KLRB1*, and (**F**, **L**) stratified by *LAT*. Low expression is marked in blue, and high expression is marked in red. The *p*-values were calculated using the Log rank test.

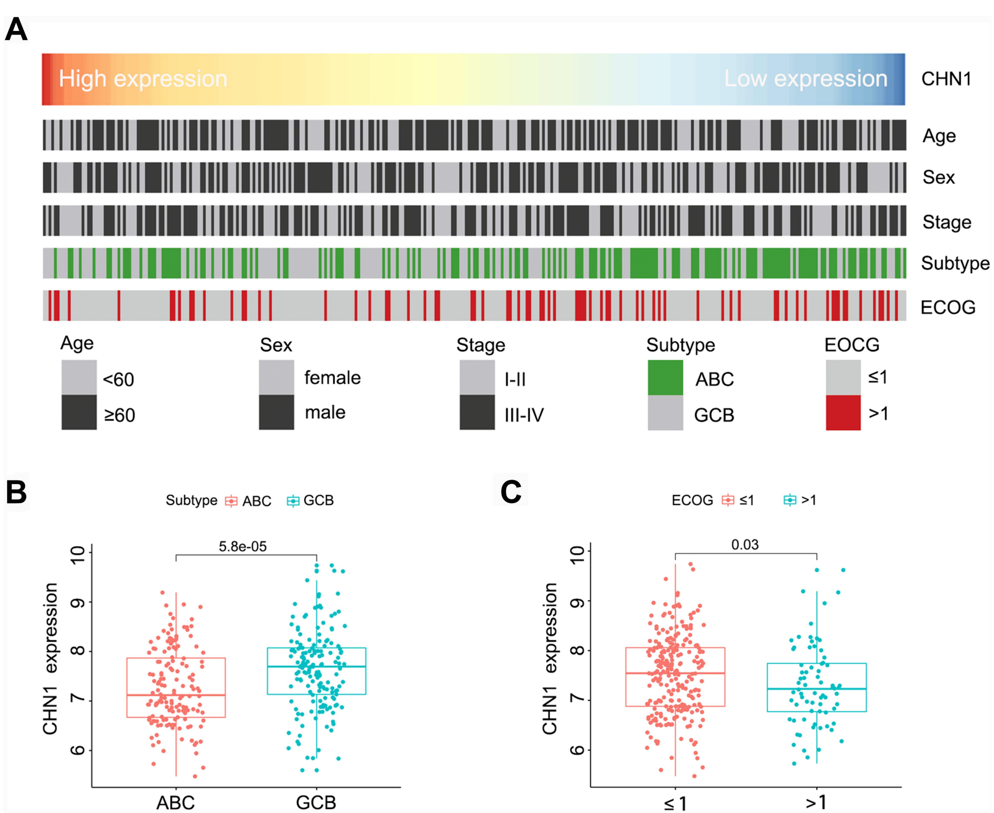

Figure 4 The correlation between *CHN1* expression and clinical features in GSE10846. (**A**) Association between clinical features and *CHN1* expression. (**B**, **C**) Distribution of *CHN1* expression in patients stratified by the molecular subtype and ECOG performance status.

development.[32–36] Of note, the role and prognostic value of *CHN1* in B-NHL have never been reported before, so we focused our attention on *CHN1* in this study.

Correlations Between *CHN1* Expression and Clinical Characteristics of DLBCL

We investigated the difference in the distribution of clinical characteristics between the high and low *CHN1* expression groups. There were obvious differences in the distribution of molecular subtype and Eastern Cooperative Oncology Group (ECOG) performance status in the GSE10846 dataset (Figure 4A); 99 (63.1%) and 130 (82.8%) patients with high *CHN1* expression were assigned to the GCB subtype and had a good ECOG performance status (ECOG < 1), respectively (Figure 4B–C, $p < 0.05$, and Supplementary Table S4). However, there were no significant differences between *CHN1* expression and clinical features stratified by age, stage, or the IPI ($p > 0.05$). We also confirmed that the majority of patients with *CHN1* overexpression in GSE117556 had the GCB subtype and an ECOG score less than 1 (Supplementary Figure S1 and Table S4). These results revealed that *CHN1* expression was significantly correlated with the molecular subtype and performance status in DLBCL patients.

Evaluation of *CHN1* Expression in Important Clinical Subgroups of DLBCL

DLBCL is divided into two important molecular subtypes: GCB and ABC. Patients with different subtypes have distinct prognoses. Therefore, we evaluated the prognostic value of *CHN1* in different molecular subtypes of DLBCL. The results revealed that a high expression level of *CHN1* was significantly related to good outcomes in patients with the GCB subtype in GSE10846 (Figure 5A, HR = 0.46 [0.26–-0.80], $p < 0.01$) and GSE117556 (Figure 5C, HR = 0.37 [0.23–0.59], $p < 0.001$). Similarly, there was a trend toward favorable prognosis for those with the ABC subtype (Figure 5B and D), but the difference was not statistically significant.

The prognosis of patients with advanced tumor stages is different from that of patients with early stages. The Kaplan–Meier curve showed a clear distinction between good and poor outcomes in stage I–II patients according to the expression level of *CHN1* (Figure 5E and G).

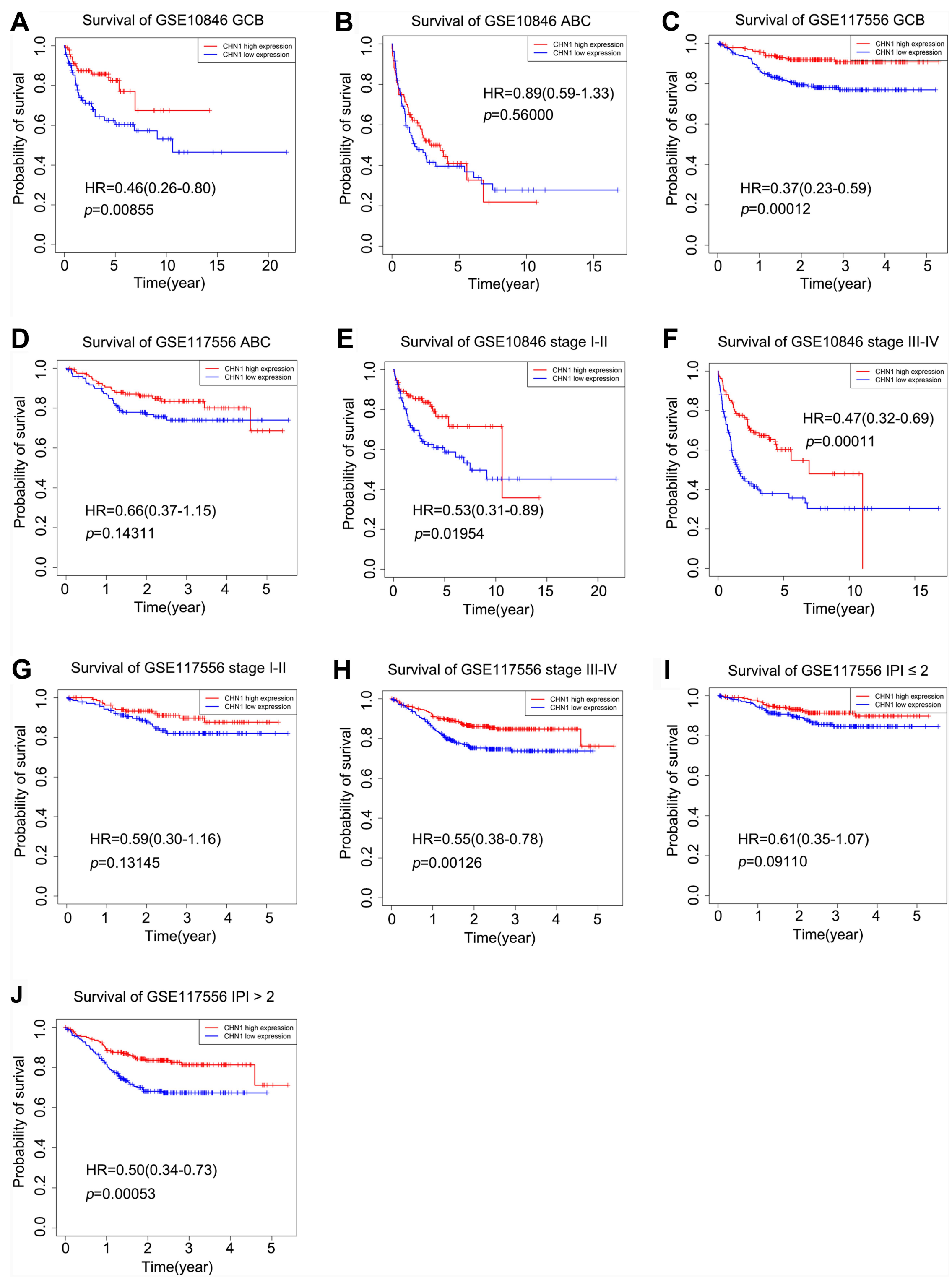

Figure 5 The prognostic significance of *CHN1* in important clinical subgroups of DLBCL. Kaplan–Meier curves of overall survival (OS) in the high *CHN1* expression and low expression groups in GSE10846 and GSE117556 with different DLBCL subtypes (**A–D**), different stages (**F–H**), and different IPI scores (**I–J**).

Furthermore, elevated expression of *CHN1* was significantly associated with a prolonged survival time of stage III–IV patients in GSE10846 (Figure 5F, HR = 0.47 [0.-32–0.69], $p < 0.001$) and GSE117556 (Figure 5H, HR = 0.55 [0.38–0.78], $p < 0.01$).

We additionally investigated the prognostic value of *CHN1* in subgroups stratified by the International Prognostic Index (IPI) score, which is a routine prognostic tool for DLBCL. It was also found that the overexpression of *CHN1* contributed to a favorable survival time in patients with an IPI > 2 (Figure 5I and J).

Taken together, these results indicate that *CHN1* expression-based classification could aid in predicting the outcomes of DLBCL patients regardless of the molecular subtype, tumor stage and IPI.

CHN1 Expression is an Independent Prognostic Factor for DLBCL Patients

Multivariate Cox regression analysis in GSE10846 showed that the expression of *CHN1* was independently correlated with OS (Table 1, HR = 0.643 [0.518–0.798], $p < 0.0001$). Consistent with these findings, *CHN1* expression was also validated as an independent factor in GSE117556 (Table 1, HR = 0.541 [0.409–0.716], $p < 0.0001$). This finding implies the potential ability of *CHN1* to predict the outcomes of patients independently.

Exploration of the Potential Biological Functions of *CHN1*

To better understand the biological functions of *CHN1*, we performed GSEA to identify significantly enriched GO terms and KEGG pathways (nominal p-value < 0.05). The high *CHN1* expression phenotype was significantly enriched in GO terms including the positive regulation of T cell cytokine production and positive regulation of T helper 1 type immune response (Figure 6A and B) and KEGG pathways including cell adhesion molecules (CAMs) and the hematopoietic cell lineage (Figure 6C and D, and Supplementary Figure S2). Therefore, we speculate that *CHN1* expression is closely related to T cell immunity and cell adhesion in DLBCL.

Accordingly, we explored whether *CHN1* expression was related to immune cell infiltration by using CIBERSORT and the LM6 signature reference profile. Interestingly, we found that the proportion of CD4+ T cells was increased in patients with high *CHN1* expression, and CD8+ T cells followed the same trend in both GSE10846 and GSE117556 (Figure 6E and F).

Discussion

We compared the gene expression profiles of FL, DLBCL and BL patients longitudinally to identify DEGs and found for the first time that high *CHN1* expression is correlated with a prolonged survival time in DLBCL. *CHN1* is located on chromosome 2q31 and encodes the protein α2-chimerin,[37–39] which is a rac guanosine triphosphatase activating protein (racGAP) that is predominantly expressed in neurons, especially in the cerebral cortex, and plays an important role in axon guidance.[40] Recent research on *CHN1* has mainly focused on Duane syndrome,[41,42] and few studies on tumors, especially lymphoma, have been reported.[43]

Table 1 Multivariable Cox Regression Analyses of *CHN1* in GSE10846 and GSE117556

Variables	GSE10846 (n=414)			GSE117556 (n=928)		
	HR	**95% CI**	***p* value**	**HR**	**95% CI**	***p* value**
CHN1 (high vs low)	0.643	0.518–0.798	< 0.0001	0.541	0.409–0.716	< 0.0001
Age	1.027	1.014–1.040	< 0.0001	1.001	0.985–1.017	0.8996
Sex (male vs female)	1.201	0.859–1.677	0.2840	0.894	0.648–1.234	0.4954
Subtype (GCB vs ABC)	0.482	0.332–0.699	0.0001	0.721	0.522–0.996	0.0473
Stage	1.405	1.193–1.654	< 0.0001	0.880	0.686–1.130	0.3165
ECOG	1.571	1.316–1.875	< 0.0001	1.338	1.061–1.668	0.0139
IPI				1.593	1.303–1.948	< 0.0001

Abbreviations: HR, hazard ratio; CI, confidence interval; GCB, germinal center B-cell-like; ABC, activated B-cell-like; ECOG, Eastern Cooperative Oncology Group performance status; IPI, International Prognostic Index.

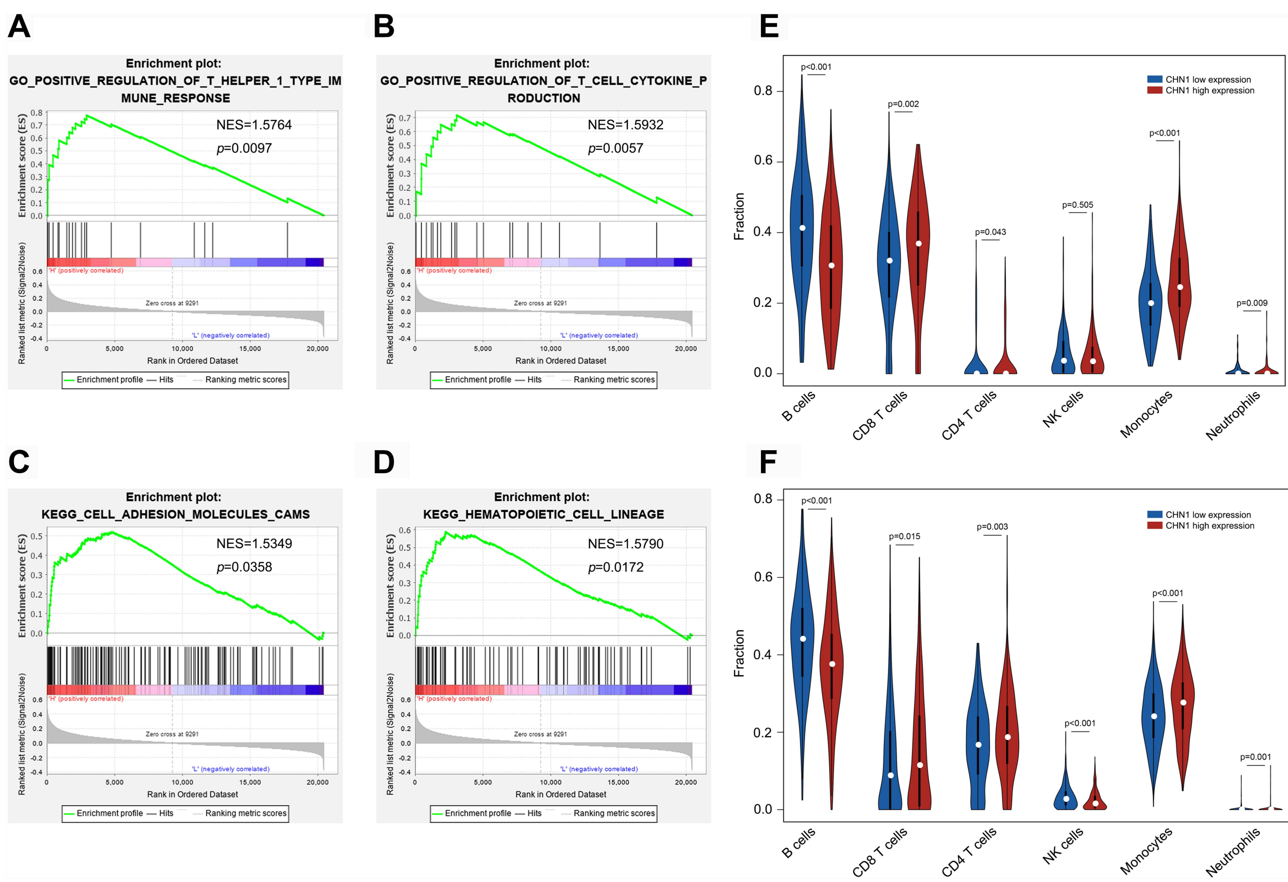

Figure 6 The potential biological functions of *CHN1*. (**A**–**D**) Enrichment plots from GSEA in GSE10846. Significantly enriched GO terms (**A**, **B**) and KEGG pathways (**C**, **D**) in the high *CHN1* expression group. (**E**, **F**) The proportion of immune infiltrating cells in the high *CHN1* expression and low expression groups in GSE10846 (**E**) and GSE117556 (**F**).

Here, we observed that the expression of *CHN1* gradually increased from highly aggressive BL to indolent FL and was inversely correlated with the aggressiveness of B-NHL. Then, we explored the difference in the distribution of clinical characteristics between the high and low *CHN1* expression groups and found that the majority of patients with *CHN1* overexpression were assigned to the GCB subtype rather than ABC subtype. ABC subtype is characterized by mutations in the B cell receptor and Toll-like receptor pathways, as well as NF-κB pathway.[44,45] As for GCB subtype, oncogenic PI3K/AKT activation is a mutational pathway.[46] Interestingly, the findings of GSEA revealed that high *CHN1* expression phenotype was significantly enriched in PI3K pathway in both GSE10846 and GSE117556 (Supplementary Figure S3). Accordingly, these results suggest that high *CHN1* expression is involved in PI3K/AKT pathway in GCB subtype of DLBCL. However, further experiments are needed to directly test this hypothesis.

Furthermore, our data showed that the high expression phenotype of *CHN1* was significantly enriched in the regulation of cell adhesion and the immune response, as well as with high infiltrating levels of CD8+ and CD4+ T cells. Combined with previous analyses, we speculate that the overexpression of *CHN1* acts as a protective factor and might be associated with a high degree of infiltration by CD8+ and CD4+ T cells. Some studies have suggested that the paucity of CD4+ T and CD8+ T cell infiltration is related to poor survival in B-NHL patients,[47–49] which is in accordance with our findings. However, we also acquired a contradictory result: the expression of *CHN1* was positively correlated with monocyte infiltration, which should be inferred as a predictor of a favorable outcome, but several studies have shown that increased monocyte/macrophage infiltration correlates with a poor prognosis.[50–53] This inconsistent issue has yet to be interpreted deeply, and further research is needed.

In our study, several limitations await to be addressed. First, this was a retrospective study, and more prospective studies are needed to confirm our results. Second, the prognostic value of *CHN1* must be validated in real clinical samples. Third, further experiments exploring the role of *CHN1* in the proliferation and invasion of lymphoma cells remain to be conducted.

Conclusion

In conclusion, we found that *CHN1* expression was an independent prognostic factor and had prognostic ability in important clinical subgroups. We believe that *CHN1* can be a convincing marker to predict the prognosis of DLBCL.

Abbreviations

GSEA, Gene Set Expression Analysis; NHL, Non-Hodgkin lymphoma; B-NHL, B-cell non-Hodgkin lymphoma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; BL, burkitt lymphoma; GEO, Gene Expression Omnibus; LLMPP, Lymphoma/Leukemia Molecular Profiling Project; DEGs, differentially expressed genes; ECOG, Eastern Cooperative Oncology Group performance status; GCB, germinal center B-cell-like; ABC, activated B-cell-like; IPI, International Prognostic Index.

Data Sharing Statement

The datasets analyzed in this study are available in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and LLMPP (http://llmpp.nih.gov).

Funding

This work was supported by grants from the Beijing Committee of Science and Technology (No. Z181100001718162).

Disclosure

The authors declare that they have no competing interests.

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