Biomarker potential of nuclear Nrf2 activation in the ABC subtype of diffuse large B-cell lymphoma

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Received July 26, 2024; Accepted October 7, 2024

DOI: 10.3892/ol.2024.14776

Abstract. Diffuse large B-cell lymphoma (DLBCL) is an aggressive B-cell lymphoma characterized by distinct subtypes and heterogeneous treatment outcomes. Oxidative stress and the dysregulation of related regulatory genes are prevalent in DLBCL, prompting an investigation into the nuclear factor erythroid 2-related factor 2 (Nrf2)-kelch-like ECH-associated protein 1 (Keap1) signaling pathway and associated genes. The present study assessed pathological specimens and clinical data from 43 newly diagnosed patients with DLBCL, comparing the associations and correlations between the expression of Nrf2, Keap1, microtubule-associated protein 1 light chain 3β (LC3B) and nitrotyrosine and the activated B-cell (ABC) and germinal center B-cell (GCB) subtypes of DLBCL using immunohistochemistry and digital image analysis software. Nuclear Nrf2 activation was observed in 33.3% of patients with DLBCL ABC, demonstrating a higher prevalence of hepatitis B surface antigen positivity, calcium ions and significant body weight loss ($P<0.05$). Total Nrf2 expression was associated with the DLBCL GCB subtype and inversely correlated with Keap1 expression in the DLBCL ABC subtype. Furthermore, a positive correlation was demonstrated between Nrf2 and LC3, indicating that total Nrf2 is inhibited by Keap1 and regulates LC3 expression. The ABC subtype was also associated with lower white blood cell counts and more frequent chemotherapy courses than the GCB subtype. These findings suggest that nuclear Nrf2 could be a biomarker for DLBCL clinical diagnosis.

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Key words: diffuse large B-cell lymphoma, nuclear factor erythroid 2-related factor 2, activated B-cell, germinal center B-cell, kelch-like ECH-associated protein 1, nitrotyrosine, microtubule-associated protein 1 light chain 3β

Introduction

Diffuse large B-cell lymphoma (DLBCL) is an aggressive malignant lymphoma and despite significant therapeutic advances in recent years, relapsed/refractory DLBCL occurs in 30-40% of patients due to DLBCL morphological and molecular heterogeneity (1-4). According to the World Health Organization, DLBCL subgroups are classified based on gene

expression profiling or cell of origin (5) and primarily stratified into germinal center B-cell (GCB) and activated B-cell (ABC) subtypes. In comparison with the GCB subtype of DLBCL, the ABC subtype is associated with a poor prognosis, with a high expression of BCL2, cMYC and BCL6, and a typical progression of clinical features (6,7). Despite the effectiveness of lenalidomide, BTK and PI3K inhibitors (8-13), ABC-type DLBCL still displays an inadequate response to standard immunotherapy (6). Furthermore, although the pathogenesis of these two DLBCL subtypes involves different mutated genes and activated signaling pathways, with corresponding drugs already available, the mechanisms remain unclear and require investigation.

Oxidative stress and related regulatory genes are considered hallmarks of cancer progression and therefore can be used to assess disease course and prognosis. The Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway is a typical antioxidant stress pathway that exhibits abnormalities in several human malignant tumors, such as breast cancer, lung cancer, liver cancer, thyroid cancer, ovarian cancer and gastric cancer (14-17). Nrf2 is a transcriptional regulatory factor for antioxidant stress and is induced by oxidative stress to enter the nucleus to activate downstream antioxidant genes to protect cells from oxidative and electrophilic stress. Conversely, upregulation of Nrf2 activity within cancer cells suppresses drug-induced reactive oxygen species (ROS) production, leading to the development of drug resistance, thereby facilitating cancer cell survival and proliferation (18). Recent studies have reported that Nrf2 is overexpressed in cancer cells, indicating that it may serve an oncogenic role in carcinogenesis (15). Moreover, due to its dual role, Nrf2 and its antagonist Keap1 have become the subject of debate regarding their specific roles in preventing or promoting tumor progression (18). Exploring the related gene expression of this pathway is of great significance for cancer prevention and treatment. In addition, previous studies have also reported the association between Nrf2 and autophagy. The autophagy receptor p62 is also a target of Nrf2, whereby induction of autophagy leads to increased p62 levels. Consequently, p62 interacts with Keap1, activating Nrf2, further promoting cancer cell survival (19-22).

Currently, there is limited data regarding the impact of Nrf2 expression in different DLBCL subtypes on subsequent treatment or clinical outcomes. Therefore, the present study assessed the gene expression of *NFE2L2* (Nrf2), *KEAP1* (Keap1) and *MAP1LC3B* (LC3B), as well as ROS, in the cells of different subtypes (ABC and GCB) of newly diagnosed patients with DLBCL. Subsequently, the present study analyzed the correlation between these expression profiles and clinical pathological data, as well as the correlations among diverse genes to assess the impact of varying gene expression on DLBCL subtypes to identify potential prognostic biomarkers.

Materials and methods

Public databases for gene expression analysis. DLBCL samples from the Cancer Genome Atlas (TCGA) (https://www.cancer.gov/ccg/research/genome-sequencing/tcga) and the Genotype-Tissue Expression (GTEx) (https://gtexportal.org/home/) databases were analyzed using Gene Expression Profiling Interactive Analysis 2 (http://gepia2.cancer-pku.cn/) and the University of Alabama at Birmingham Cancer data analysis Portal (https://ualcan.path.uab.edu/) software (23,24). The gene expression data included *NFE2L2*, *KEAP1* and *MAP1LC3B* and the value |log2FoldChange|>1 and q values <0.01 were considered to indicate differential expression. These databases provide tumor/normal differential expression analysis to aid in the analysis of RNA-sequencing data.

Study design. Following approval by the Institutional Review Board of Kaohsiung Medical University Chung-Ho Memorial Hospital [Kaohsiung, Taiwan; approval nos. KMUHIRB-E(I)-20210119 and KMUHIRB-E(I)-20220298], pathological specimens of patients diagnosed with DLBCL at Kaohsiung Medical University Chung-Ho Memorial Hospital between July 2015 and December 2021 were collected, along with clinical and biochemical laboratory data for subsequent analysis. A total of 43 specimens were obtained and DLBCL subtypes were classified as ABC or GCB subtypes using immunohistochemistry (IHC) analysis (25). The inclusion criteria included the following: i) Histopathologically-con firmed DLBCL; ii) age of ≥18 years; and iii) pathological tissue sections measuring ≥1x1x5 mm. The exclusion criteria included the following: i) Concurrent malignancies; ii) aged of <18 years; and iii) adequate pathological specimens were unavailable or too small for analysis.

IHC. The staining process was automated using a BOND-MAX Automated IHC Staining System (Leica Biosystems) following a standardized protocol (26). Briefly, pre-existing formalin-fixed paraffin-embedded blocks were sectioned to 4-μm thick, deparaffinized with xylene at 72˚C and pre-treated for permeabilization using Epitope Retrieval Solution 1 (citrate, pH 6.0; Leica Biosystems) at 100˚C for 20 min. Hydroperoxide blocking was performed for 5 min using the Bond Polymer Refine Detection Kit (Leica Biosystems Newcastle Ltd, United Kingdom), incubated with the following primary antibodies at room temperature for 30 min: Primary antibodies targeting Nrf2 (EP1808Y; monoclonal; 1:50; cat. no. ab62352; Abcam), Keap1 (1B4; monoclonal; 1:150; cat. no. ab119403; Abcam), LC3B (polyclonal; 1:100; cat. no. ab63817; Abcam) and nitrotyrosine (39B6; monoclonal; 1:100; cat. no. sc-32757; Santa Cruz Biotechnology, Inc.). Afterward, the tissue was incubated with the secondary antibody from the BOND Polymer Refine Detection Kit (Leica Biosystems) at 25˚C for 15 min. Polymer incubation lasted for 15 min before development with 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) chromogen for 10 min. The specimens were counterstained with hematoxylin at 25˚C for 5 min. Positive and negative controls consisted of squamous cell carcinoma tissues. The staining of cytoplasmic Nrf2, Keap1, LC3B and nitrotyrosine was visualized using the TissueFAXS PLUS system (version 4.2; TissueGnostics GmbH) and the Zeiss Observer microscope with a 20X objective lens (Ziess GmbH). HistoQuest software (version 4.0; TissueGnostics GmbH) was used to quantify cytoplasmic Nrf2, Keap1, LC3B, nitrotyrosine and hematoxylin staining (Fig. S1). Regions of interest (ROIs) within each slide were selected for analysis, with ≥3 representative areas measured for consistency. Positive cell

expression within the ROIs was quantified as %. Staining intensity was assessed by assigning arbitrary numbers based on grayscale pixel conversion, with the numbers of DAB-positive and hematoxylin-positive events used for optimization. This approach ensured robust analysis of immunohistochemically stained samples adhering to established protocols for accurate interpretation. Due to the limitations of imaging software in accurately distinguishing between nuclear and cytoplasmic Nrf2, pathologists qualitatively assessed the nuclear expression of Nrf2 in DLBCL using optical microscopy. A positivity threshold of ≥10% was used to define a sample as positive.

Cell culture. Human DLBCL cancer cell lines, U2932 (Guangzhou Ubigene Biosciences Co., Ltd.) and HT (cat. no. 60486; Bioresource Collection and Research Center, Taiwan) were cultured in RPMI-1640 supplemented with 10% FBS (Merck KGaA), 2 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc.), 10 mM HEPES (BioConcept AG), 1 mM sodium pyruvate (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in 5% CO_2 at 37˚C. A total of $2x10^6$ cells were initially seeded into a 75T flask with 10 ml medium. Every 3-4 days, the cells and medium were transferred to a 15 ml centrifuge tube and centrifuged at 150 x g for 5 min at 25˚C. After centrifugation, the supernatant was carefully discarded and half of the cells were resuspended in fresh medium.

Reverse transcription-quantitative (q)PCR. Total RNA was isolated from the U2932 and HT cell lines using the TOOLSmart RNA Extractor reagent (cat. no. DPT-BD24; BIOTOOLS Co., Ltd.) and 1 μg RNA reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (cat. no. 4368814; Thermo Fisher Scientific, Inc.) at 25˚C for 10 min, followed by 37˚C for 120 min, 85˚C for 5 min and maintenance at 4˚C. Each 20 μl reaction contained 2x SYBR Green Master Mix (cat. no. A46012; Applied Biosystems; Thermo Fisher Scientific, Inc.), 1 μl forward and reverse primers (*KEAP1* forward: 5'-CGTAGCCCCCATGAAGCA-3' and reverse: 5'-ACTCCACACTGTCCAGGAACGT-3'; *GAPDH* forward: 5'-GCACCACCAACTGCTTAGCA-3' and reverse: 5'-TCTTCTGGGTGGCAGTGATG-3') and 2 μl cDNA. qPCR was performed using the QuantStudio real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the thermocycling conditions were as follows: 95˚C for 1 min, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 60 sec; 65˚C for 10 sec for melting curves; and 40˚C for 30 sec for cooling. Target gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method with GAPDH used as the internal control (27,28). The PCR reactions were performed in triplicate.

Statistical analysis. Statistical analyses were performed using SPSS software (version 19; IBM Corp.). Pearson's correlation coefficient was used to assess the correlation between Nrf2, Keap1, LC3B and nitrotyrosine. Continuous variables were compared between groups using an unpaired t-test. Fisher's exact test or the χ^2 test were used to evaluate the association of categorical variables. Multiple logistic regression models were also used to assess the association between nuclear Nrf2 and clinical characteristics. Independent variables with P<0.05 in univariable analysis were selected in the multivariable analysis. A forward selection approach determined the final multivariable model. Continuous data are presented as mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Differential distribution of Nrf2 in subtypes and stages of DLBCL. Nrf2 is a crucial gene involved in mitigating ROS and is present in several cancers (Fig. S2). In DLBCL, the *NFE2L2* (Nrf2) mRNA level also demonstrated a significantly higher expression, with transcripts per million (TPM) of 23.29, compared with 19.69 TPM in normal tissues, as revealed by analysis of data from the TCGA and GTEx databases (Fig. 1A). Furthermore, a higher proportion of Nrf2-positive cancer cells was demonstrated in the advanced cancer stages (Ann Arbor stages 3 and 4) (29), where 60.88% cells were Nrf2-positive, compared with 51.26% in the early cancer stages (stages 1 and 2) of DLBCL, as determined by IHC analysis (Fig. 1B). Furthermore, when comparing the subtypes of DLBCL, it was demonstrated that the GCB subtype (84.20%) had a significantly higher frequency of Nrf2-positive cancer cells than the ABC subtype (45.32%) (P<0.0001; Fig. 1C). This trend persisted across disease stages, where the GCB subtype had a significantly greater proportion of Nrf2-positive cells compared with the ABC subtype in both the early stages (GCB, 75.98%; and ABC, 37.78%) and the late stages (GCB, 91.25%; and ABC, 49.69%) (Fig. 1D). The results revealed that Nrf2 was prominently distributed in the advanced stages of DLBCL and showed a higher frequency in the GCB subtype compared with that in the ABC subtype.

Active Nrf2 is predominantly located in the nucleus of the DLBCL ABC subtype. Nrf2 functions as a transcription factor activated within the cell nucleus (30). Therefore, the present study further assessed the nuclear expression of Nrf2 in DLBCL. Fig. 2A and B present images captured at x40 magnification, of Nrf2 localization in the nucleus and cytoplasm of DLBCL cells, respectively. Notably, the analysis revealed that nuclear Nrf2 was exclusively present in the ABC subtype, accounting for 33.3% of cases (n=30), whilst no nuclear Nrf2 was observed in the GCB subtype (n=13; Fig. 2C).

KEAP1 mRNA and protein expression in advanced cancer stages and the DLBCL ABC subtype. A comparative assessment of mRNA levels from the public databases of TCGA and GTEx revealed higher *KEAP1* expression in tumor samples compared with that in controls. Specifically, *KEAP1* exhibited an average expression of 54.07 TPM in tumor samples, significantly elevated compared with 6.1 TPM in normal controls (P<0.05; Fig. 3A). Immunohistochemical staining for Keap1 also demonstrated higher expression in advanced stages (stages 3 and 4), with 87.10% Keap1-positive cells, compared with 76.66% in the early stages (stages 1 and 2; P=0.046; Fig. 3B). Additionally, the *KEAP1* expression ratios in DLBCL cell lines revealed a 4.3-fold higher expression in U2932 cells (ABC) compared with that in HT cells (GCB) when normalized to GAPDH (P<0.01; Fig. 3C). Moreover, assessment of Keap1 expression across DLBCL subtypes indicated a higher proportion of Keap1-expressing cells in tumor samples from

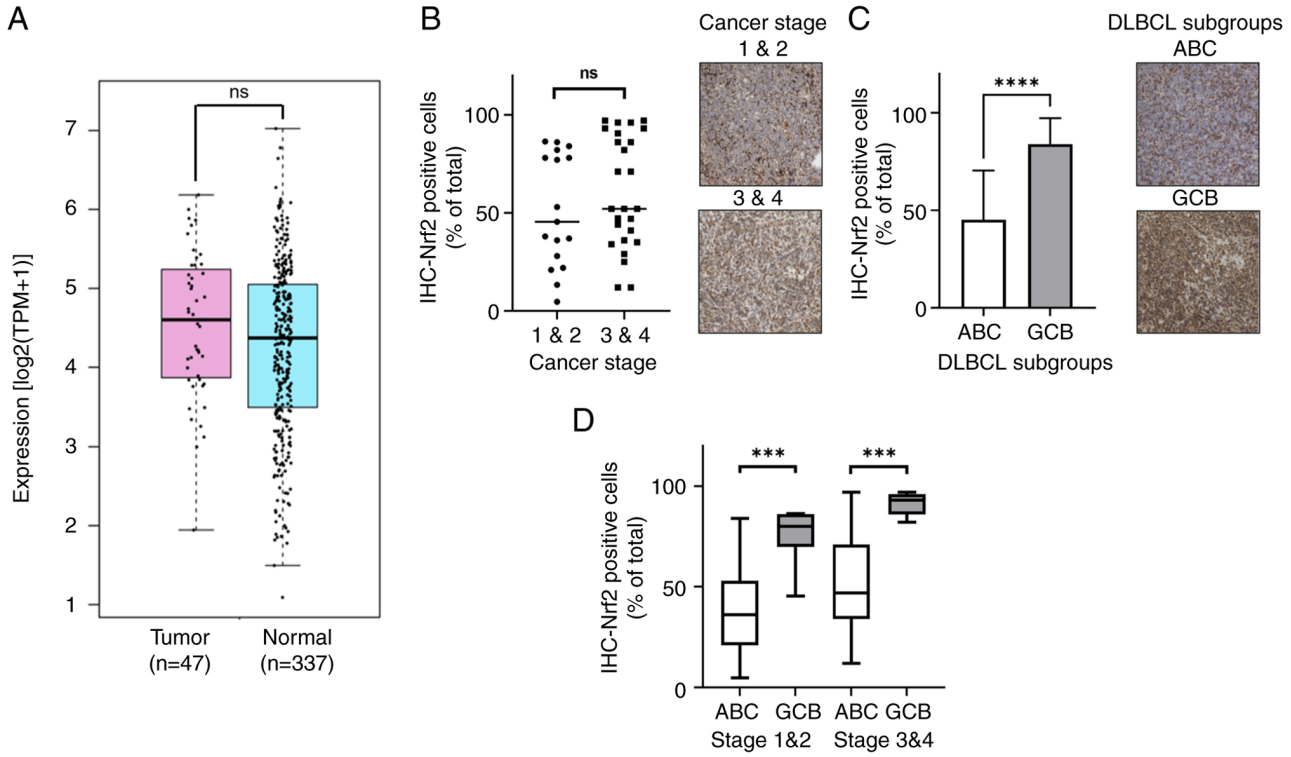


Figure 1. Nrf2 is differentially distributed in different cancer stages and subtypes of DLBCL. (A) Elevated NFE2L2 (Nrf2) mRNA levels in DLBCL tissues compared with in normal tissues. (B) Increased % Nrf2-positive cancer cells in advanced stages of DLBCL. (C) Higher frequency of Nrf2-positive cancer cells in the GCB subtype of DLBCL compared with that in the ABC subtype. (D) Higher Nrf2-positive cancer cell numbers in the GCB subtype across all stages of DLBCL. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. All IHC sample images are x20 magnification. ns, no significance; DLBCL, diffuse large B-cell lymphoma; Nrf2, nuclear factor erythroid 2-related factor 2; ABC, activated B-cell; GCB, germinal center B-cell; TPM, transcripts per million; IHC, immunohistochemistry.

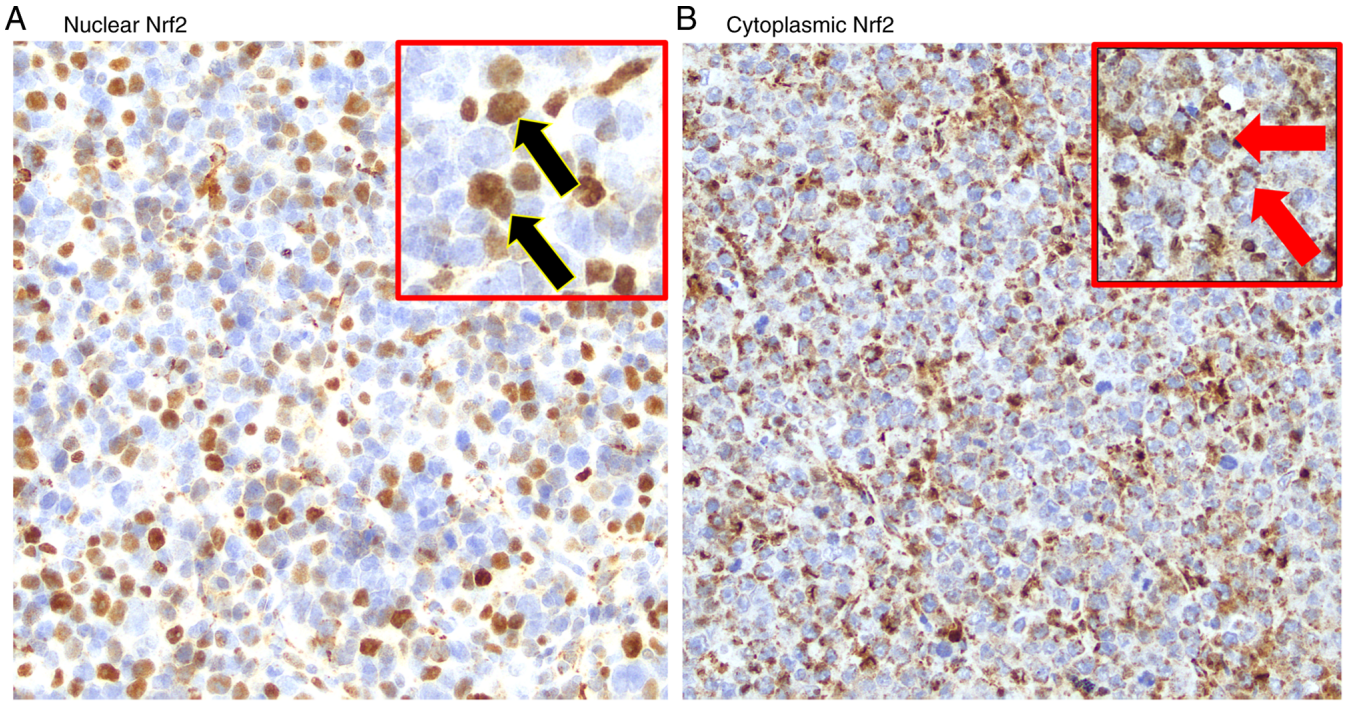
patients with ABC (78.69%) compared with those with the GCB subtype (54.86%; P=0.014; Fig. 3D).

LC3B expression and association with Nrf2 in DLBCL. Nrf2 has been implicated in autophagy (31,32), therefore the present study evaluated the expression of the autophagosome marker LC3B in DLBCL and its relationship with Nrf2. Analysis of TCGA and GTEx data revealed that MAP1LC3B (LC3B) mRNA expression was significantly elevated in DLBCL compared with normal tissues (30.58 TPM vs. 8.77 TPM; P<0.05; Fig. 4A). In IHC-stained samples, although statistical significance was not achieved, LC3B-positive cells were notably more prevalent in advanced stages (stages 3 and 4) and in the GCB subtype of DLBCL compared with that in early stages (stages 1 and 2) and the ABC subtype, respectively (Fig. 4B and C). Furthermore, a significant positive correlation was observed between Nrf2 and LC3B expression in DLBCL samples (Pearson correlation=0.345; P=0.023; data not shown). These results indicate that LC3B expression in advanced cancer stages and the GCB subtype parallels that of Nrf2.

Nitrotyrosine expression slightly increases in advanced stages and ABC subtype of DLBCL. To evaluate whether Nrf2 suppresses ROS, nitrotyrosine was used as a marker to assess ROS levels in DLBCL. IHC analysis revealed a trend of markedly increased nitrotyrosine levels in advanced stages of DLBCL compared with that in early stages (Fig. 5A). Additionally, the frequency of nitrotyrosine-positive cells was notably higher in the ABC subtype compared with that in the GCB subtype, although this difference was not statistically significant (Fig. 5B).

Clinical differences between ABC and GCB subtypes of DLBCL. Given the classification of DLBCL into ABC and GCB subtypes, a comparative analysis of the clinical data was performed to elucidate the differences between these subgroups. Table I presents the clinical characteristics of the ABC and GCB subtypes. The findings revealed that the ABC subtype was significantly associated with a lower white blood cell (WBC) count compared with those with the GCB subtype (P=0.0096). Moreover, patients in the ABC subtype underwent a significantly greater number of chemotherapy cycles compared with those with the GCB subtype (P=0.0173). Additionally, several clinical parameters, including Eastern Cooperative Oncology Group performance status (33), overall survival (OS), progression-free survival (PFS), platelet count, lactate dehydrogenase, albumin, glutamic oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), blood urea nitrogen, creatinine and ionized calcium levels, were markedly lower in the ABC subtype compared with that of the GCB subtype. These data indicate that the ABC subgroup presented with worse clinical values.

Patients with nuclear expression of Nrf2 have worse clinical biomarkers. Previous findings indicated the presence of



C

DLBCL	ABC (n=30)	GCB (n=13)
NRF2	Nucleus (+): 10 (33.3%)	Nucleus (+): 0 (0%)
	Nucleus (–): 20 (66.7%)	Nucleus (–): 13 (100%)

Nucleus (+): threshold>10%

Figure 2. Immunohistochemical staining of Nrf2 localization in DLBCL. (A) Active Nrf2 is distributed in both the nucleus and cytoplasm, covering the entire cell (black arrows). (B) Nrf2 localized in the cytoplasm surrounds the nucleus (red arrows indicate cytoplasmic Nrf2; nucleus stained with hematoxylin). (C) Nuclear Nrf2 is predominantly observed in the ABC subtype of DLBCL and is absent in the GCB subtype. All IHC sample images are x40 magnification. DLBCL, diffuse large B-cell lymphoma; Nrf2, nuclear factor erythroid 2-related factor 2; ABC, activated B-cell; GCB, germinal center B-cell.

nuclear Nrf2 in the ABC subtype of DLBCL (Fig. 2C). To further assess the impact of nuclear Nrf2 expression on patient outcomes, clinical data were collected and analyzed by dividing patients into two groups based on the presence or absence of nuclear Nrf2. The comparison revealed significant associations with several clinical characteristics (Table II). Specifically, hepatitis B surface antigen (HBsAg), body weight loss (BWL) and ionized calcium were significantly higher in the nuclear Nrf2-positive group than in the Nrf2-negative group ($P<0.05$). Additionally, there was a marked trend towards lower WBC and platelet counts in the nuclear Nrf2-positive group compared with that of the nuclear Nrf2-negative group, whilst higher levels of GOT and GPT were observed in the nuclear Nrf2-positive group compared with that of the nuclear Nrf2-negative group. These findings demonstrate that nuclear Nrf2 expression had a notable impact on the clinical biomarkers of patients with DLBCL.

Discussion

DLBCL, the most common subtype of non-Hodgkin's lymphoma in Asia, is regarded as a severe form of non-Hodgkin's lymphoma and is increasing in incidence (34). DLBCL can be further categorized into ABC and GCB subtypes based on cell of origin and gene expression analysis (35). The frequency of the ABC subtype is 60-70% in Asian countries, which is markedly higher than the 37-40% observed in Western countries, and it is associated with a worse prognosis (36,37). The distinction between ABC and GCB subtypes notably impacts the prognosis of patients with DLBCL (38), as the expression of antioxidant genes in cancer cells can attenuate the efficacy of drug therapy, leading to drug resistance and relapse (39). Despite extensive research on DLBCL, there are limited studies focusing on the association between DLBCL subtypes, antioxidant genes like Nrf2 and Keap1, and clinical data. Therefore, the present study assessed the relationship between the different DLBCL subtypes (ABC and GCB), clinical data and the expression of Nrf2, Keap1, nitrotyrosine and LC3B.

Nrf2, a transcription factor with antioxidant capabilities, activates in the nucleus and is associated with worse treatment outcomes and prognosis in cancer therapy (30). In the dataset in the present study, ~23.26% (10/43) of DLBCL tissue specimens exhibited nuclear Nrf2 expression in tumor cells, particularly in the ABC subtype with worse prognosis, where it accounted for 33.33% (10/33), potentially contributing to chemotherapy resistance and worsening treatment outcomes. Previous studies have reported an association between Nrf2 expression and drug resistance in cancers, as well as its association with clinical characteristics (40). Generally, higher Nrf2 expression is associated with a worse patient prognosis,

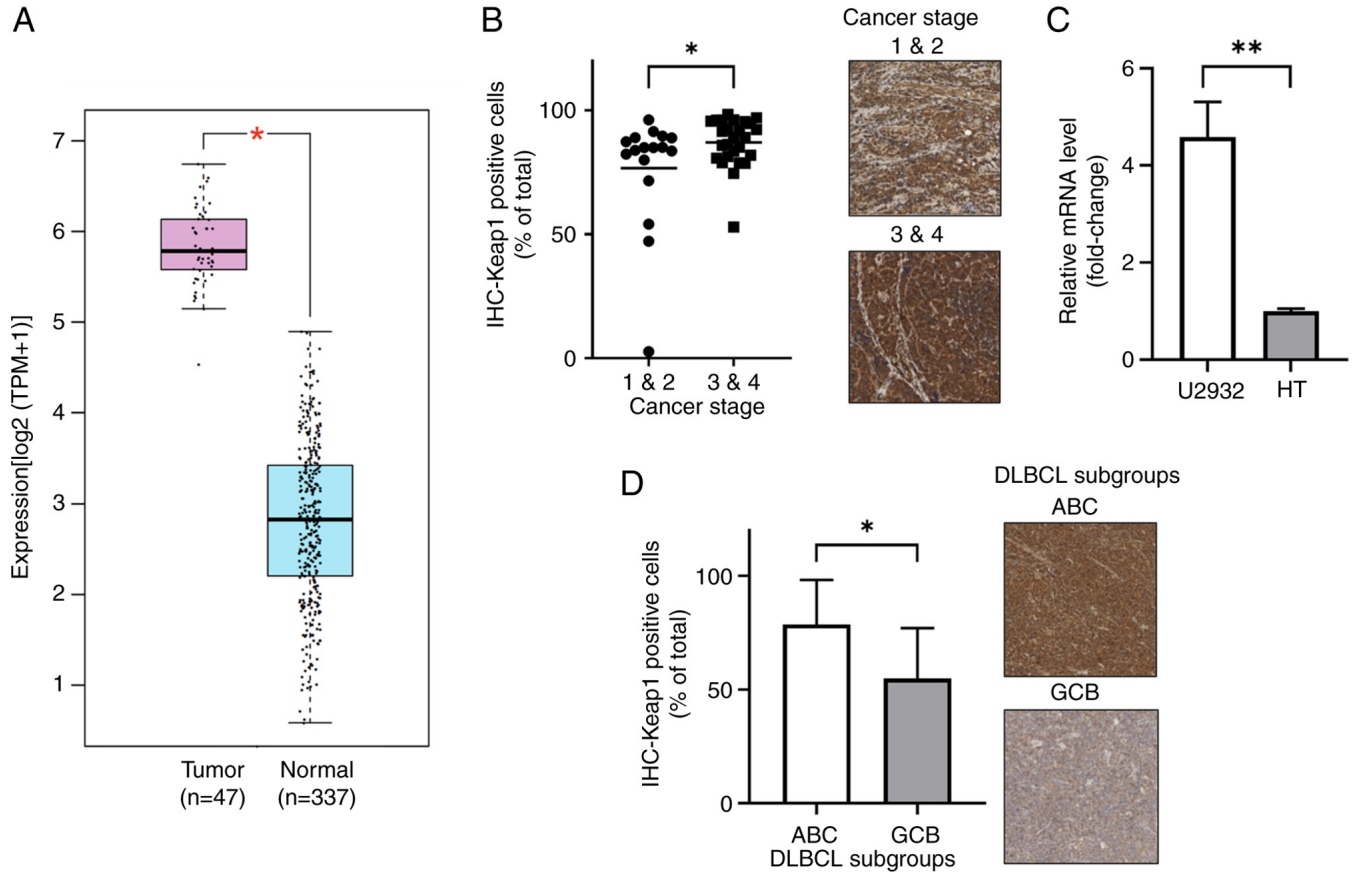


Figure 3. Keap1 expression in DLBCL, especially in the advanced staged and ABC subtype. (A) KEAP1 mRNA is significantly upregulated in DLBCL compared with that in the controls in The Cancer Genome Atlas and Genotype-Tissue Expression databases. (B) Significantly higher Keap1 expression in the advanced stages of DLBCL than in the early stages. (C) Significantly higher Keap1 expression in patients with DLBCL of the ABC subtype than the GCB subtype. (D) KEAP1 mRNA expression in DLBCL cell lines (HT and U2932). *P<0.05; **P<0.01. All IHC sample images are x20 magnification. DLBCL, diffuse large B-cell lymphoma; Keap1, kelch-like ECH-associated protein 1; ABC, activated B-cell; GCB, germinal center B-cell; TPM, transcripts per million; IHC, immunohistochemistry.

suggesting its potential as a cancer biomarker (30,39,41). The clinical data analysis in the present study revealed a higher prevalence of HBsAg in patients with nuclear Nrf2 expression, indicating a potential link between Nrf2 activation and HBsAg presence in DLBCL. Previous studies have also reported that Hepatitis B virus (HBV) regulatory proteins hepatitis B virus X protein (HBx) and large hepatitis B virus surface protein activate Nrf2 via the c-Raf and MEK pathways, thereby protecting HBV-positive cells from oxidative damage (42-44). Moreover, previous studies reported that a marked reduction in HBsAg release was associated with decreased Nrf2 activity (45,46). Conversely, other research suggested that during HBV infection, ROS production was induced, with HBsAg contributing to ROS formation. HBx further activated Nrf2 by increasing its protein levels and enhancing nuclear localization. However, excessive Nrf2 expression markedly suppressed HBV core promoter activity, leading to reduced viral replication (47,48). These findings suggest that nuclear Nrf2 activation, associated with HBsAg presence in DLBCL, may protect cancer cells from oxidative stress, indicating its potential as a biomarker for identifying patients with HBsAg-positive DLBCL. Furthermore, in the present study, patients with DLBCL with nuclear Nrf2 expression exhibited significant weight loss, suggesting that Nrf2 activation may contribute to this outcome. Previous studies using a diabetic mouse (db/db) model reported that Nrf2 activation by the inducer CDDO-Im markedly suppressed high-fat diet-induced obesity and alleviated diabetes, leading to weight loss. This is a process reversed by Nrf2 gene disruption (49,50). This mechanism involves insulin secretion by β-cells, where ROS generated from glucose metabolism activate Nrf2. In turn, Nrf2 stimulates the expression of downstream genes such as glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase in the pentose phosphate pathway, producing NADPH, which enhances insulin secretion, glucose metabolism and energy expenditure, ultimately affecting body weight (51,52). The association between nuclear Nrf2 expression and weight loss in patients with DLBCL suggests that Nrf2 activation may influence metabolism, reinforcing its potential as a biomarker for assessing clinical outcomes.

Under normal conditions, the transcription factor Nrf2 is bound by the Keap1-dependent E3 ubiquitin ligase complex, which includes Keap1, Cullin 3 and RING box protein 1, along with an E2 ubiquitin-conjugating enzyme. This interaction facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2. However, under stress conditions, sensor cysteine residues within Keap1 are modified, allowing Nrf2 to escape degradation, translocate to the nucleus and initiate its

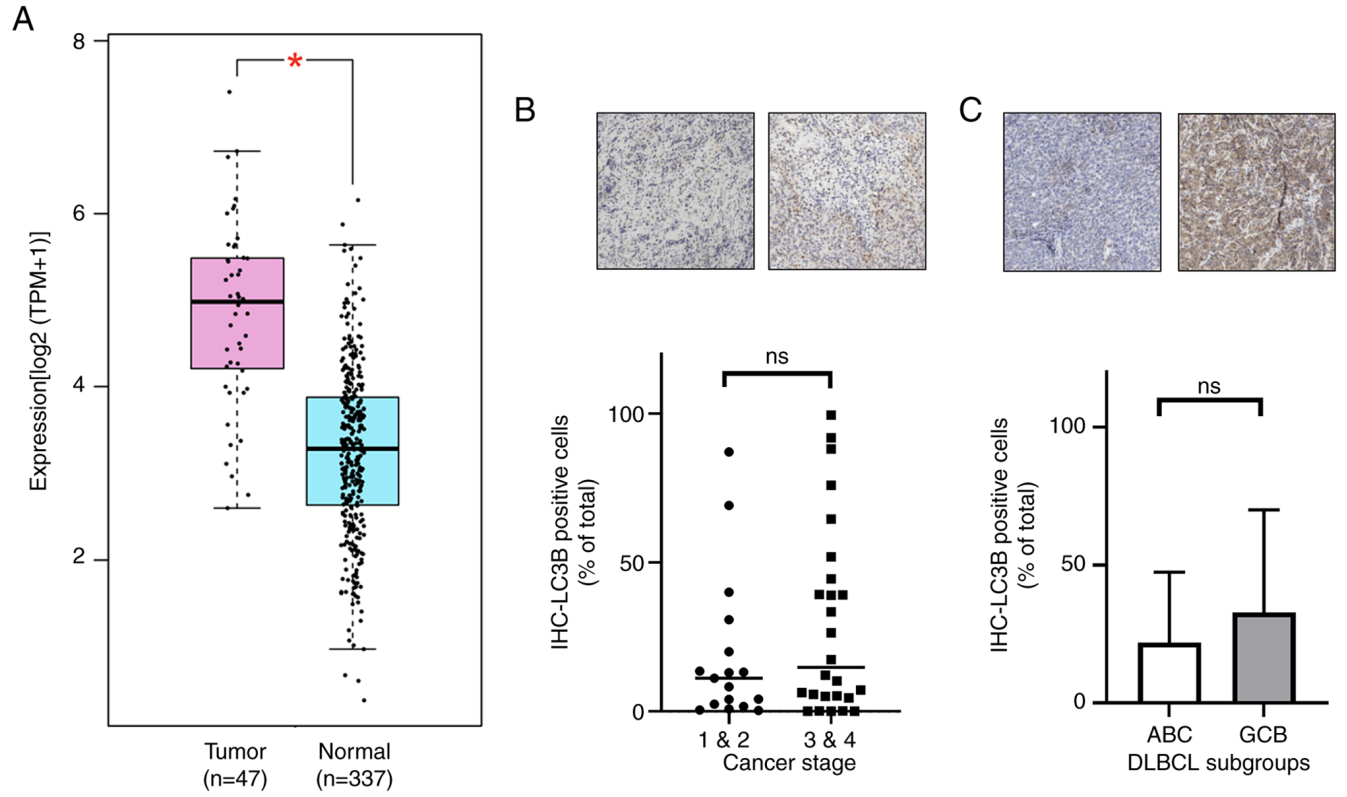


Figure 4. Increased LC3B (encoded by MAP1LC3B) expression in DLBCL. (A) Elevated *MAP1LC3B* mRNA expression in DLBCL compared with controls in The Cancer Genome Atlas and Genotype-Tissue Expression databases. (B) LC3B expression across the early and advanced stages of DLBCL. (C) LC3B expression in the DLBCL GCB and ABC subtypes. All IHC sample images are x20 magnification. ns, no significance; DLBCL, diffuse large B-cell lymphoma; TPM, transcripts per million; IHC, immunohistochemistry; MAP1LC3B, microtubule-associated protein 1 light chain 3β.

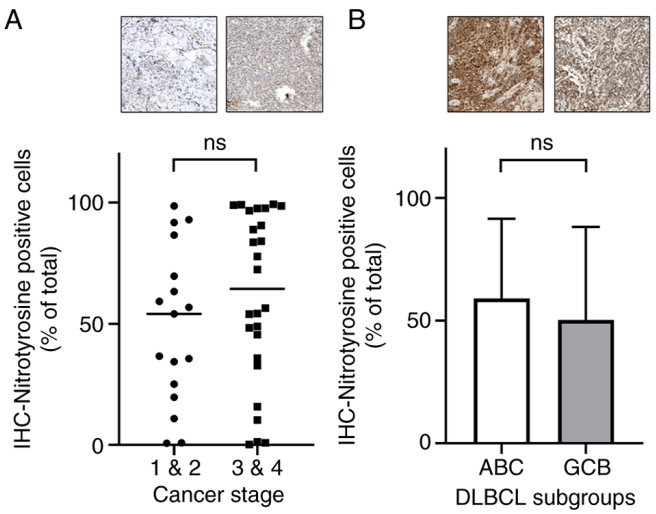


Figure 5. Nitrotyrosine expression in different DLBCL stages and subgroups. (A) Nitrotyrosine-positive cells were more prevalent in the advanced stages of DLBCL. (B) Higher frequency of nitrotyrosine-positive cells was observed in the ABC subtype of DLBCL. All IHC sample images are x20 magnification. ns, no significance; DLBCL, diffuse large B-cell lymphoma; IHC, immunohistochemistry.

antioxidant transcriptional program (53). The findings of the present study, consistent with those reported by Yi *et al* (41), demonstrated significant Keap1 expression in DLBCL, particularly in advanced stages of the disease. In the ABC subtype, which is characterized by elevated oxidative stress (54), Nrf2 predominantly localized to the nucleus and was positively associated with HBsAg expression and BWL, suggesting increased oxidative stress in this subtype. Furthermore, despite elevated Keap1 expression in ABC subtype, its regulatory function appeared to be impaired, resulting in enhanced nuclear translocation of Nrf2. This dysregulation likely contributed to the activation of aberrant metabolic and stress response pathways in ABC, negatively influencing clinical outcomes. In contrast, Nrf2 was primarily confined to the cytoplasm in the GCB subtype, indicating that the inhibitory function of Keap1 remained intact. This suggests that the Keap1-Nrf2 axis was more effectively regulated in GCB, allowing for a more controlled cellular response to oxidative stress. These findings highlight substantial differences in the regulation of the Nrf2-Keap1 pathway between the ABC and GCB subtypes of DLBCL. In ABC, the compromised inhibitory function of Keap1 may serve a pivotal role in disease progression by promoting oxidative stress-induced cellular damage, whereas in GCB, Keap1 retained its suppressive role, potentially contributing to the less aggressive disease phenotype observed in this subtype. Previous studies have reported abnormal expression of Keap1 and Nrf2 in solid tumors, possibly due to mutations in the *NFE2L2* or *KEAP1* genes, preventing Keap1 from binding to Nrf2, thereby allowing Nrf2 to enter the nucleus and be activated (55,56). Additionally, abnormalities in *NFE2L2* and *KEAP1* in DLBCL may not solely result from genetic mutations but may involve other factors. Both GCB and ABC DLBCL cell lines exhibit autophagy-dependent characteristics when treated with autophagy inhibitors, with no difference in LC3B

Table I. Basic clinical characteristics of the diffuse large B-cell lymphoma activated B-cell and germinal center B-cell subtypes.

Clinical characteristic	ABC (n=30)	GCB (n=13)	P-value
Sex			0.1854
Male	13	9	
Female	17	4	
Age, years	58.5	62.5	0.4313
Ann Arbor stage			0.4970
1-2	10	4	
3-4	20	9	
ECOG performance status			0.3607
<2	25	10	
≥2	3	3	
N.D.	2	0	
OS, months	33.0±30.8	34.1±43.0	0.9240
PFS, months	28.2±32.0	34.1±43.0	0.6215
HBsAg			0.6972
Positive	6	2	
Negative	19	10	
N.D.	5	1	
HBcAb			0.4340
Positive	15	8	
Negative	10	2	
N.D.	5	3	
HCV			0.6594
Positive	4	3	
Negative	21	9	
N.D.	5	1	
B symptoms[b]			0.2953
Positive	14	4	
Negative	11	8	
N.D.	5	1	
WBC, μl	6,189.6±1,912.2	9,545.0±5,543.8	0.0096[a]
Platelet, x$10^3/\mu$l	230.5±118.9	257.7±129.1	0.5311
LDH, U/l	378.6±437.6	673.5±1091.9	0.2459
β-2 microglobulin, μg/l	435.9±483.1	306.9±215.9	0.4055
Albumin, g/dl	3.9±0.6	3.9±0.4	0.8598
GOT, U/l	31.6±18.9	45.9±46.2	0.1860
GPT, U/l	26.9±24.7	30.1±19.4	0.6960
Blood urea nitrogen, mg/dl	16.7±9.6	19.4±14.4	0.4981
Creatinine, mg/dl	1.38±2.1	1.52±2.2	0.8558
Ionized calcium, mg/dl	4.68±0.4	4.94±0.4	0.0945
Chemotherapy courses	5.93±2.25	3.75±3.05	0.0173[a]

Data are presented as n or mean ± standard deviation. [a]P<0.05. [b]B symptoms: Fevers >38˚C for at least 3 consecutive days, night sweats and body weight loss >10% during the 6 months prior to diagnosis. ABC, activated B-cell; GCB, germinal center B-cell; ECOG, Eastern Cooperative Oncology Group; OS, overall survival; PFS, progression-free survival; HBsAg, hepatitis B surface antigen; HBcAb, Hepatitis B core antibody; HCV, Hepatitis C virus; WBC, white blood cell; LDH, lactate dehydrogenase; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; N.D., not detected.

expression between subtypes (57). The results of the present study also indicated a positive correlation between Nrf2 and LC3B. As a major autophagy-related gene, LC3B expression is regulated by p62, which promotes autophagy generation and regulates LC3B expression, whilst also activating Nrf2 by bypassing Keap1 through non-canonical pathways (31,32,58).

Table II. Clinical analysis of nuclear factor erythroid 2-related factor 2 expression in diffuse large B-cell lymphoma.

Clinical characteristic	Nuclear Nrf2-positive	Nuclear Nrf2-negative	P-value
Subtype			0.0196[a]
ABC	10	20	
GCB	0	13	
HBsAg			0.0487[a]
Positive	4	4	
Negative	4	25	
N.D.	2	4	
BWL			0.0421[a]
Yes	6	9	
No	2	20	
N.D.	2	4	
WBC, μl	6471.3±1290.6	7500.0±4220.8	0.5043
Platelet, x$10^3/\mu$l	201.1±83.8	239.8±136.6	0.3207
GOT, U/l	37.1±29.5	36.0±31.5	0.9621
GPT, U/l	35.4±38.0	25.9±17.1	0.3044
Ionized calcium, mg/dl	5.1±0.4	4.7±0.4	0.0134[a]

Data are presented as n or mean ± standard deviation. [a]P<0.05. Nrf2, nuclear factor erythroid 2-related factor 2; ABC, activated B-cell; GCB, germinal center B-cell; HBsAg, hepatitis B surface antigen; BWL, body weight loss; WBC, white blood cell; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; N.D., not detected.

In summary, abnormalities in the *NFE2L2* or *KEAP1* genes may lead to Nrf2 activation without Keap1 inhibition and may also be regulated by non-canonical pathways involving sequestosome 1 (*SQSTM1*/p62). These possibilities suggest the activation of nuclear Nrf2 and cytoplasmic Keap1 expression in ABC subtype DLBCL. Nrf2 induction is associated with ROS generation. In the present study, immunohistochemical analysis revealed no differences in ROS levels across different cancer stages or DLBCL subtypes. This may be due to ROS being detected by nitrotyrosine, which specifically represents tyrosine nitration and may not comprehensively reflect total ROS, potentially introducing measurement bias (59). Additionally, the similar ROS expression indicates that the amount of ROS generated under oxidative stress is consistent across different DLBCL subtypes. Alternatively, whilst the ROS levels appear comparable, the activated Nrf2 in the ABC subtype may have already suppressed a portion of the ROS. Consequently, the original ROS levels in the ABC subtype would be higher; however, the retrospective data of the present study cannot demonstrate this.

Patients with DLBCL of the ABC subtype have a worse prognosis, as confirmed by the analysis of patient medical records in the present study. Despite being older on average, patients with the DLBCL GCB subtype demonstrated better OS and PFS compared with those with the ABC subtype (Table I). Furthermore, patients with GCB exhibited higher WBC counts and required fewer chemotherapy sessions, indicating better treatment outcomes in patients with DLBCL GCB than in patients with DLBCL ABC. The worse prognosis of patients with the DLBCL ABC subtype compared with those with the GCB subtype may be attributed to differences in nuclear Nrf2 expression. Whilst the GCB subtype exhibited cytoplasmic accumulation of Nrf2, this inactive form did not influence the disease progression in GCB patients. This hypothesis is supported by the clinical data in Table II, which demonstrates that patients with nuclear Nrf2 expression have worse clinical outcomes.

The present study has certain limitations. The small sample size of patients with the GCB subtype limits the generalizability of the conclusions regarding Nrf2 expression. The difficulty in obtaining pathological specimens and the lack of complete clinical records had also contributed to the inability of the present study to collect a larger sample size. Additionally, in Asian countries, the ratio of the DLBCL ABC subtype to GCB subtype is ~2:1 (37), which further explains the limited number of GCB subtype samples in the present study. Despite the smaller sample size, the present study demonstrated certain trends in Nrf2 expression in patients with ABC subtype DLBCL were associated with clinical data, warranting further investigation. However, there was no associated demonstrated between distinct genes, such as Nrf2 and Keap1, and there were no statistically significant differences in OS between the DLBCL ABC and GCB subtypes in the cohort due to the limited sample size and salvage therapy. Typically, patients with the ABC subtype receive salvage therapy after disease progression, and the diverse treatment options, including novel pharmacological agents, hematopoietic stem cell transplantation and cellular therapy, may also contribute to the absence of statistically significant differences in OS between the subtypes (9,10,13). Furthermore, whilst IHC staining was performed using automated staining equipment with control groups for comparison, the staining intensity remains subject to interpretation. Moreover, gene expression levels were analyzed by calculating the % stained cells, and human subjective decisions were still required to define the regions of interest for calculating changes in cell staining even

with automated quantification using the HistoQuest software. Efforts were made to reduce bias by randomly selecting ≥3 regions of interest for averaging. Future directions should focus on efficient computer-based identification to reduce subjectivity and potential bias. In future studies, multi-center and prospective research should be performed to collect a larger sample size and more diverse patient populations. This will enable a more detailed analysis of the differences in Nrf2 expression in DLBCL subtypes and clinical characteristics, and will help mitigate the impact of sample size on the interpretation of results, thereby validating and expanding upon the findings of the present study.

In conclusion, the proportion of Nrf2-positive cells was predominantly distributed in advanced stages of DLBCL and the GCB subtype, correlating with LC3B expression, whereas activated nuclear Nrf2 was exclusively detected in the nuclei of cells within the ABC subtype. Patients with nuclear Nrf2-positive DLBCL were more frequently associated with clinical symptoms such as HBsAg positivity and significant BWL. Additionally, Keap1 expression increased with disease progression and was significantly elevated in the GCB subtype. These findings suggest that the oxidative stress marker Nrf2 may serve as a potential biomarker for the ABC subtype in DLBCL, aiding in the identification of therapeutic targets.

Acknowledgements

The authors would like to thank Dr Ming-Yen Lin from the Division of Medical Statistics and Bioinformatics, Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung Medical University for assisting with correctly applying statistical methods. The authors would also like to thank Ms. Li Li Lin from the Biobank, Kaohsiung Medical University Hospital, Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung Medical University, for her assistance with the IHC stain.

Funding

The present study was supported by the Kaohsiung Medical University Hospital [grant nos. KMUH109-M907, KMUH111-1R16, KMUH111-1M17, KMUH112-2M12, KMUH112-2M13 and KMUH-DK(B)111002-3] and the Ministry of Health and Welfare (grant no. MOHW112-TDU-B-212-144006).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CMH, KCC and HHH designed the present study and contributed to the conceptualization. HHH contributed to the project administration. CMH and CHY cultured the DLBCL cell lines. CHY, YT and LCH performed qPCR. CHY, YT, SYH and LCH scanned the IHC-stained samples using TissueFAXS PLUS. SYH, LCH, SYK, CEH, YT and KCC quantified the IHC staining through HistoQuest analysis. SFC, HCW, TJY, JSD, MHW, TYH and YCL collected the clinical data. SYK, HCW, KCC and HHH organized the patient data. SFC, HCW, TJY, JSD, MHW, TYH and YCL analyzed the patient data and performed statistical analyses. SYK, CEH, SYH, LCH, KCC and HHH revised and edited the manuscript. CMH, YT and HHH validated the data. CMH wrote the original draft. HHH and CMH confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was performed in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Kaohsiung Medical University Chung-Ho Memorial Hospital [approval nos. KMUHIRB-E(I)-20210119 and KMUHIRB-E(I)-20220298]. Due to the retrospective nature of the study, the requirement for informed patient consent was waived.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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