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ORIGINAL PAPER

Haematological Malignancy – Clinical

Molecular characterization of diffuse large B-cell lymphomas associated with hepatitis C virus infection

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Summary

Hepatitis C virus (HCV)-associated diffuse large B-cell lymphoma (DLBCL) displays peculiar clinicopathological characteristics, but its molecular landscape is not fully elucidated. In this study, we investigated the clinicopathological and molecular features of 54 patients with HCV-associated DLBCL. The median age was 71 years. An underlying marginal zone lymphoma component was detected in 14.8% of cases. FISH analysis showed rearrangements involving *BCL6* in 50.9% of cases, *MYC* in 11.3% and *BCL2* in 3.7%. Lymph2Cx-based assay was successful in 38 cases, recognizing 16 cases (42.1%) as ABC and 16 cases as GCB subtypes, while six resulted unclassified. ABC cases exhibited a higher lymphoma-related mortality (LRM). Next-generation sequencing analysis showed mutations in 158/184 evaluated genes. The most frequently mutated genes were *KMT2D* (42.6%), *SETD1B* (33.3%), *RERE* (29.4%), *FAS* and *PIM1* (27.8%) and *TBL1XR1* (25.9%). A mutation in the NOTCH pathway was detected in 25.9% of cases and was associated with worst LRM. Cluster analysis by *LymphGen* classified 29/54 cases within definite groups, including BN2

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in 14 (48.2%), ST2 in seven (24.2%) and MCD and EZB in four each (13.8%). Overall, these results indicate a preferential marginal zone origin for a consistent subgroup of HCV-associated DLBCL cases and suggest potential implications for molecularly targeted therapies.

KEYWORDS
diffuse large B-cell lymphoma, hepatitis C virus, Nanostring, NGS

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) accounts for nearly 40% of mature B-cell neoplasms. Hepatitis C virus (HCV) infection, estimated to affect about 70 million people worldwide, has been consistently associated with DLBCL, especially in countries with a high prevalence of infection.[1] Several studies have demonstrated the presence of typical features in patients with HCV-associated DLBCL, such as older age, extra-nodal involvement, elevated serum lactate dehydrogenase (LDH) and high International Prognostic Index (IPI).[2–4] Moreover, it was observed that the proportion of 'transformed' DLBCL, in particular from a previous marginal zone lymphoma (MZL), is substantially higher among HCV-positive patients (up to 30%) compared to HCV-negative DLBCL.[5] Furthermore, up to a quarter of de novo HCV-associated DLBCL cases display histological evidence of a residual low-grade lymphoma component, consistent with MZL features.[6] In recent years, the use of novel direct-acting anti-virals (DAAs), with the achievement of sustained virological response (SVR) in nearly all treated cases with negligible toxicity,[7] has demonstrated to induce lymphoma regression in about half of patients with HCV-associated indolent lymphomas. These cases are largely represented by MZL, for which DAAs are now recommended as first-line treatment.[8–11] In the DLBCL setting, although data are less conclusive, the use of DAAs, either concurrently or subsequently to standard immunochemotherapy, has been suggested to potentially ameliorate the long-term outcome.[12–14]

In the last few years, the genetic landscape of DLBCL has been thoroughly investigated, and novel molecular classifications have been introduced. However, these studies only evaluated patients who were HCV-negative or with unknown HCV status.[15–18] Consequently, data regarding the molecular characteristics of HCV-associated DLBCL remain limited. A case–control study reported that patients with de novo HCV-associated DLBCL lack *BCL2* translocations and exhibit differential expression of genes involved in innate immune regulation and apoptotic pathway compared to HCV-negative controls.[19] Only two small series (less than 20 cases) described cell-of-origin (COO) distribution by gene expression profiling (GEP) in HCV-associated DLBCL, with conflicting results (*activated B-cell-like* [ABC] in 47%[19] and 18%[14]). With regards to mutational analysis, through targeted sequencing of hotspots of 11 genes in 46 patients, we have previously shown that about a quarter of HCV-associated DLBCL display mutations in the NOTCH pathway,[6] which is also typically mutated in MZL.[20,21] Finally, a recent study evaluated a cohort of HCV-positive lymphoma, including eight cases of DLBCL, by whole exome sequencing (WES) and described novel putative genes with pronounced specificity for HCV-associated DLBCL.[22]

In this study, we performed a comprehensive characterization of the COO and molecular landscape of a well-annotated series of HCV-associated DLBCL cases and applied the *LymphGen* classification for the first time in this setting.[17]

METHODS

We retrospectively evaluated 54 patients diagnosed with DLBCL and positive HCV serology between 2000 and 2021 at the Divisions of Hematology of Fondazione IRCCS Policlinico San Matteo, Pavia and Ospedale di Circolo e Fondazione Macchi, Varese (Italy). The study was approved by the local Ethics Committee of the two participating institutions. Data collection and analysis were conducted by the tenets of the Declaration of Helsinki of 1964, as revised in 2000.

Histological diagnosis of DLBCL was established by expert pathologists at each participating institution and reviewed in accordance with the 5th edition of the World Health Organization Classification of Haematolymphoid Tumours.[23] HCV positivity was defined as the presence of serum antibodies against HCV using an enzyme-linked immunosorbent assay (ELISA) at the time of lymphoma diagnosis. Human immunodeficiency virus-positive patients were excluded. The serum HCV-RNA load was determined by a reverse transcriptase polymerase chain reaction.

The disease stage was assessed according to the Lugano Classification.[24] IPI,[25] revised-IPI (R-IPI)[26] and hepatitis C prognostic score (HPS)[4] were calculated for all patients with available data.

Histology, immunohistochemistry and FISH

The scoring of CD10, MUM1 and BCL6 staining and subsequent classification as *germinal center B-cell-like* (GCB) or non-GCB was performed by Hans algorithm-based immunohistochemistry.[27]

FISH analysis was performed on formalin-fixed and paraffin-embedded (FFPE) tissue sections and was carried out using dual colour break-apart probes for *BCL2* (18q21 region), *BCL6* (3q27) and *MYC* (8q24) (Supplementary data).

Gene expression profiling by Lymph2Cx-based assay (Nanostring)

Gene expression analysis was conducted through the Lymph2Cx-based assay with NanoString Technologies, as previously described.[28] Each sample was reported as one of the two molecular subtypes, that is ABC and GCB, or Unclassified (Supplementary data).

Mutational analysis

Targeted NGS was performed on DNA extracted from FFPE tissue biopsies using a probe capture-based strategy. The probe panel included 184 genes (coding exons and splice sites) recurrently mutated in B-cell neoplasms. The gene panel included a cluster of histone genes, major histocompatibility complex genes, transcription factor genes and chromatin-remodelling genes. To generate libraries, we used the Illumina DNA prep with enrichment (Supplementary data).

Based on the information retrieved from the KEGG and REACTOME databases, each mutated gene was assigned to a specific lymphoma-enriched pathway.

To calculate the probability of a given DLBCL sample belonging to one genetic subtype according to *Wright* et al classification,[17] we used the web-based implementation of the *LymphGen* algorithm (https://llmpp.nih.gov/lymphgen/index.php).

Statistical analysis

Demographic and clinical characteristics were summarized using descriptive statistics. Overall survival (OS) was defined as the time from diagnosis to death or last follow-up. Progression-free survival (PFS) was defined as the time from diagnosis to progression, relapse, death or last follow-up, whichever occurred first. Lymphoma-related mortality (LRM) was defined as the time from diagnosis to death due to lymphoma or treatment-related toxicity.[29] OS and PFS were estimated by the Kaplan–Meier method, and comparison between curves was tested via the log-rank test and proportional hazard Cox regression model. Results from Cox models were reported in terms of Hazard Ratios (HR) with its 95% confidence interval (95% CI). LRM was estimated in a competing-risk approach (considering events not related to lymphoma as competing events) with the Kalbfleisch and Prentice method. The comparison of LRM between groups of patients was evaluated by the Pepe&Mori test and by the Fine & Gray regression model. Results from Fine & Gray models were reported in terms of subhazard ratios (sHR) with its 95% CI.

A two-tailed type I error was set at 5%. Statistical analysis was performed using Stata 18 (StataCorp. 2023. *Stata Statistical Software: Release 18*. College Station, TX: StataCorp LLC).

RESULTS

Baseline characteristics, treatment and outcome

Clinical, histological and virological features of 54 HCV-positive patients affected by DLBCL are reported in Tables 1 and 2 and Table S1. The median age was 71 years (33–84), with 36 patients (66.7%) aged ≥65 years. Twenty-two patients (40.7%) presented with extra-nodal localization (bone in eight cases, liver in five, gastrointestinal tract in four, lung, soft tissue and upper airways in two, kidney, breast, pleura, parotid and skin in one), including six patients with involvement of multiple extra-nodal sites. Spleen involvement was detected in 22 patients (40.7%), including eight with exclusive splenic involvement (diagnosis performed on splenic tissue by splenectomy), six with splenic and nodal involvement, four with coexisting liver localization and four with other extra-nodal sites.

A history of previous indolent lymphoma was reported in four patients (7.4%) (three follicular lymphoma, one low-grade lymphoma not otherwise specified), two of which displayed a residual component of indolent lymphoma in the diagnostic DLBCL sample. In eight additional cases (14.8%), including four with splenic, two with extra-nodal (lung and soft tissue) and two with exclusive nodal involvement, a co-existing infiltrate of small to medium-sized monocytoid B cells resembling histological features of MZL was detected.

HCV-RNA was detectable at baseline in 52 patients (96.3%), while it was negative in two patients previously treated with anti-viral therapy (AT). All patients were HBsAg negative, 15 were HBcAb positive and HBV-DNA was negative in all patients.

Overall, 21 out of 46 patients with available data (45.6%) received a course of AT, including six (28.6%) with interferon (IFN)-based regimens and 15 (71.4%) with direct-acting anti-virals (DAA). Six patients received AT before lymphoma onset, two of which achieved SVR (one with IFN, one with DAA); the remaining four, all treated with IFN-based regimens, did not. Among the remaining 15 patients, one was treated with DAA concurrently with immunochemotherapy, 13 received DAA and 1 IFN after a median of 15.5 months from lymphoma treatment completion (range 1–82). Overall, 16 patients achieved SVR after AT, including all 15 patients treated with DAAs (100%) and one out of six treated with IFN-based regimens (16.7%).

Three patients were lost to follow-up early after diagnosis, and one received only palliative care. Overall, 50 patients were treated with curative-intent regimens (Table 1).

In an intention-to-treat analysis, with a median follow-up of 8.2 years (IQR: 4.6–10.6), median OS and PFS were 4.4 years (95% CI: 1.5–8.0) and 2.4 years (95% CI: 0.9–5.5) respectively (Figure 1).

FISH and cell-of-origin

FISH analysis showed rearrangements involving *BCL6* in 27 patients (50.9%), *MYC* in six (11.3%) and *BCL2* in two

TABLE 1 Baseline clinical characteristics of 54 HCV-positive patients affected with diffuse large B-cell lymphoma.

	Patients, *N*	Total data available
Age, median (range)	71 (33–84; IQR: 61.9–77)	54
Sex (M/F)	27/27 (50%/50%)	54
Involved site		
Nodal	32 (59.3%)	54
Extra-nodal	4 (7.4%)	
Nodal and extra-nodal	18 (33.3%)	
Spleen involvement	22 (40.7%)	54
Bone marrow involvement	6 (16.2%)	36
B symptoms	23 (42.6%)	54
Stage		
I	6 (11.1%)	54
II	14 (25.9%)	
III	18 (33.3%)	
IV	16 (29.7%)	
ECOG PS		
0–1	14 (25.9%)	54
2–4	40 (74.1%)	
IPI score		
Low	13 (25.0%)	52
Low-intermediate	10 (19.2%)	
High-intermediate	18 (34.6%)	
High	11 (21.2%)	
R-IPI score		
Good	2 (3.7%)	54
Intermediate	22 (40.7%)	
Poor	30 (55.6%)	
LDH > UNL	36 (66.6%)	54
β_2-microglobulin > UNL	36 (66.6%)	54
ALT > UNL	19 (35.8%)	53
Albumin <3.5 g/dL	11 (23.9%)	46
HCV genotype		
1	10 (33.3%)	
2	16 (53.4%)	30
3	4 (13.3%)	
HPS		
Low	11 (23.9%)	46
Intermediate	18 (39.1%)	
High	17 (37.0%)	
Anti-HBc positive	15 (28.3%)	53
Cryoglobulins	5 (21.7%)	23
First-line therapy		
R-CHOP-like	28 (56.0%)	50
CHOP-like	19 (38.0%)	
Other (VACOP-B, IPAD)	4 (8.0%)	
First-line response		
CR	32 (64.0%)	50
PR	2 (4.0%)	
SD/PD	14 (28.0%)	
Death during therapy (toxicity)	2 (4.0%)	

Abbreviations: ALT, alanine transaminase; CR, complete response; ECOG, Eastern Cooperative Oncology Group; F, female; HCV, hepatitis C virus; HPS, Hepatitis C Prognostic Score; IPI, International Prognostic Index; LDH, lactate dehydrogenase; M, male; PD, progressive disease; PR, partial response; PS, performance status; R-IPI, revised International Prognostic Index; SD, stable disease; UNL, upper normal limit.

(3.7%), of which one had DLBCL transformed from previous follicular lymphoma. Only three patients (5.6%) exhibited translocation of *MYC* concurrent with *BCL2* (1 case) or *BCL6* (2 cases).

Hans algorithm classified 22 out of 45 revised samples as GCB (48.9%) and 23 (51.1%) as non-GCB.

Lymph2Cx-based assay was successful in 38 out of 54 cases (70.4%) and recognized 16 cases each (42.1%) as ABC or GCB subtypes, while six cases (15.8%) resulted Unclassified (Table 2; Tables S1 and S2). The concordance between the Hans immunohistochemistry algorithm and GEP-based methods was 60% (Cohen's K = 0.57, 95% CI: 0.27–0.87). Notably, both cases with *BCL2* translocation, all cases with concurrent translocation of *MYC* and *BCL2* or *BCL6*, and four out of five available cases (80%) with *MYC* rearrangements were GCB by Nanostring.

Mutational profile

A targeted NGS panel of 184 genes was applied in 51 samples, while in the first three patients, we used a panel including 144 genes. The prevalence of gene mutations is shown in Figure 2 (complete data in Figure S1 and Tables S3 and S4).

The panel revealed 801 somatic mutations, with at least one mutation in 158 out of the 184 analysed genes. Notably, 97 genes (52.7%) were recurrently mutated in at least 5% of patients, suggesting that the panel was highly informative.

All samples harboured at least one oncogenic variant with a median mutation load (MML) of 13 mutated genes per case (range 2–26; IQR: 9–16) and a median of 14 mutations per case (range 2–32; IQR: 9.25–19). The most frequently mutated gene was the epigenetic regulator *KMT2D* ($n = 23$ patients, 42.6%). Specifically, we identified 29 *KMT2D* mutations (double mutations in six patients), the majority represented by potentially inactivating mutations, that is, stop-gain mutations (11; 37.9%), frameshift mutations ($n = 10$; 34.5%) and splice donor variant ($n = 1$). The remaining mutations included missense variant mutations ($n = 6$; 20.7%) and disruptive in-frame insertions ($n = 1$).

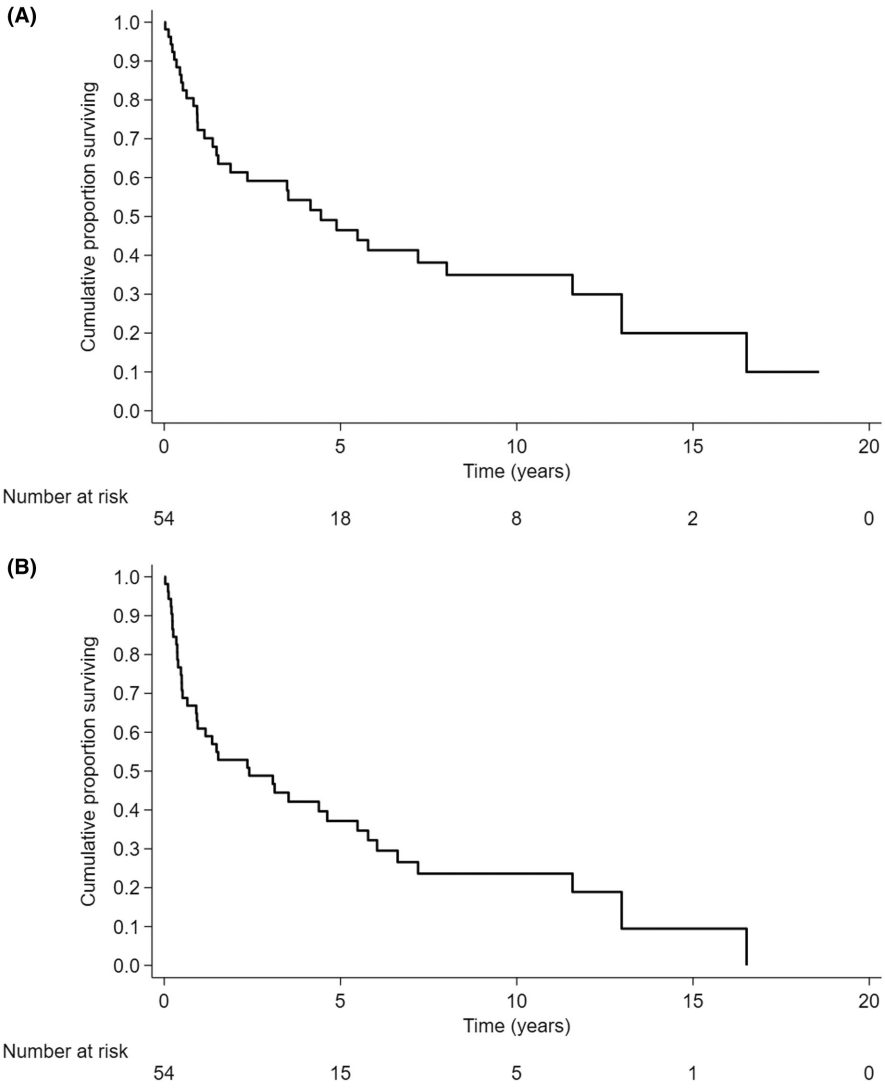

FIGURE 1 Progression-free survival (PFS) (A) and overall survival (OS) (B) of 54 patients affected by HCV-positive diffuse large B-cell lymphoma.

The second most frequently mutated gene was *SETD1B* ($n = 17$, 33.3%), with 16 out of 19 mutations (84.2%) represented by frameshift substitutions, while the remaining were missense mutations ($n = 2$) and splice variants ($n = 1$).

Other frequently mutated genes were *RERE* (29.4%), *FAS* and *PIM1* (27.8% each), *TBL1XR1* (25.9%), *BCL11A* (25.5%), *SGK1* (22.2%), *ZFP36L1* (19.6%), *USH2A* (18.5%) and *TNFAIP3* (14.8%). *TP53* was mutated in nine patients (16.7%). Interestingly, we did not observe a higher tumour mutation burden in *TP53*-mutated cases compared with *TP53*-wild-type cases (MML = 15 vs. 13, $p = 0.27$). The *MYD88* gene presented missense substitutions in eight cases (14.8%), including L265P in five and S219C in three. Finally, a mutation in the epigenetic regulators *CREBBP* and *EZH2* (missense substitution in the SET domain) was detected in 16.7% and 3.7% of cases respectively.

Compared to two large published series of HCV-negative DLBCL,[15,18] mutational frequencies of *FAS*, *TBL1XR1, BCL11A* and *ZFP36L1* resulted higher in our series. Moreover, higher mutation rates of *SETDB1, RERE, SGK1* and *USH2A* were detected with respect to Schmitz's series,[15] while *KMTD2D, ARID1A* and *KLF* were more frequently mutated compared with Lacy's one[18] (Table S5).

As a next step, we integrated the mutated genes in predefined signalling pathways or lymphomagenesis-related gene sets (Table S6). Overall, the pathways exhibiting the highest number of mutated genes were those involved in epigenetic regulation (94.4% of patients), cell cycle or apoptosis (75.9%) and BCR/NF-kB signalling (70.4%). Additionally, 51.8% of patients harboured mutations in genes involved in immune regulation, 25.9% in both B-cell development and NOTCH pathways (*NOTCH2* in seven patients, *NOTCH1* in one; *SPEN* in three; *DTX1* in six) and finally 18.5% in the JAK/STAT pathway (Figure S2).

Of note, regarding the *BCR* signalling pathway, we did not identify any primary mutations affecting BTK or genes of the 'alternative' NF-*k*B pathway (*BIRC3, TRAF3, MAP3K14*). We identified mutations affecting *CD79B* (14.8%) or genes involved in the *BTK* downstream signalling pathway, such as *PRKCB* (11.8%), *CARD11* and BCL10 (7.4%) and genes for the

TABLE 2 Baseline characteristics of 54 HCV-positive patients affected with diffuse large B-cell lymphoma.

	Patients, *N* (%)	Total data available
COO (by *Hans algorithm*)		
GCB	22 (48.9%)	45
non-GCB	23 (51.1%)	
COO (by *NanoString*)		
GCB	16 (42.1%)	38
ABC	16 (42.1%)	
Unclassified	6 (15.8%)	
Proliferation index by Ki-67		
30–50%	18 (38.3%)	47
60–80%	17 (36.2%)	
≥80%	12 (25.5%)	
FISH analysis		
BCL2 translocation	2 (3.7%)	54
BCL6 translocation	27 (50.9%)	53
MYC translocation	6 (11.3%)	53
Cluster analysis		
BN2	14 (48.3%)	29[a]
EZB	4 (13.8%)	
MCD	4 (13.8%)	
ST2	7 (24.1%)	

Abbreviations: COO, cell-of origin; FISH, fluorescence in situ hybridization.

[a]25 cases (46.3%) were not classified by cluster analysis ('Unclassified').

IKK complex (5.6%). No concurrent mutations in *CARD11* and *BCL10* were identified.

The *LymphGen* tool classified into defined clusters 29 of 54 patients (53.7%; Figure 3A), including 14 in BN2 (48.3%), seven in ST2 (24.1%) and four each in MCD and EZB subtypes (13.8%; Figure 3B). Notably, all assessable MCD cases (3/3) displayed an ABC signature, while all EZB tumours (3/3) showed a GCB COO. Conversely, ST2 and BN2 subtypes comprised mixed COO subgroups (Figure 4). Interestingly, among seven cases exhibiting low-grade component with definite subtype allocation, five showed BN2 profile, one ST2 (transformed from previous nodular lymphocyte-predominant Hodgkin lymphoma) and one EZH2 (transformed from previous FL). Of note, the patient with cutaneous DLBCL was classified as MCD.

Outcome prediction

OS and PFS were predicted by standard IPI and R-IPI (all $p < 0.003$). In analysing the role of AT in the outcome, improved OS was observed in the 15 patients who received DAA, while a landmark analysis from the time of SVR did not show a statistically significant survival benefit (HR = 0.72, 95% CI: 0.11–4.54, $p = 0.73$). The coexistence of a low-grade infiltrate did not predict a significantly different OS (HR = 0.56, 95% CI: 0.2–1.3, $p = 0.180$).

Considering FISH data, the only patient with *MYC/BCL2* rearrangement had a rapidly fatal outcome, while both patients with *MYC/BCL6* translocation responded to first-line R-CHOP but died in CR due to lymphoma-unrelated events. Considering in detail the four patients with transformed DLBCL from a previous indolent lymphoma, two died from disease progression and two were alive and in CR at the last follow-up. The prognostic analyses did not change if excluding these latter cases.

Compared to patients with ABC COO, those with GCB or Unclassified COO by Nanostring tended to have better OS (HR = 0.5, 95% CI: 0.2–1.1, $p = 0.089$). However, a significantly better LRM was detected in GCB/Unclassified patients compared to ABC ones (sHR = 0.3, 95% CI: 0.1–0.9, $p = 0.040$, Figure S3). Conversely, the COO by Hans algorithm did not predict any difference in OS ($p = 0.552$). We did not identify a difference in OS according to he number of mutations per patient (≤11, 12–17, ≥18, $p = 0.353$).

When considering NGS-defined signalling pathways, at least one mutation in the B-cell development gene set (*PRDM1, BCL6, EBF1, IKZF3, ETS1*), detected in 14 patients, was associated with an improved OS (unadjusted HR = 0.3, 95% CI: 0.1–0.9, $p = 0.010$; Table S7). Conversely, at least one mutation in the NOTCH pathway (*NOTCH1, NOTCH2, SPEN, DTX1)*, detected in 14 patients, was associated with a worse LRM (unadjusted sHR = 2.49, 95% CI: 1.0–5.9, $p = 0.040$). Perhaps due to the small number of classified cases, *LymphGen*-defined subtypes did not show significantly different OS and LRM, except for the extremely detrimental outcome exhibited by the EZB cluster, which was enriched in *MYC* rearranged cases (two out of four; Figures S4 and S5).

DISCUSSION

In this study, we provided a comprehensive molecular characterization of a relatively large and homogeneous series of patients affected by HCV-associated DLBCL. We used histological revision and conventional FISH analysis for *MYC, BCL2* and *BCL6* translocations, performed GEP by Lymph2Cx assay (Nanostring)[28] as well as an extensive mutational analysis using a large NGS panel including 184 genes commonly mutated in lymphomas. Finally, we applied the novel *LymphGen* classification.[17]

A large body of studies have highlighted the distinct clinical features of HCV-associated DLBCL compared to their negative counterpart.[2–4] The baseline characteristics of our cohort are consistent with previous series concerning older age, frequent extra-nodal involvement and elevated LDH and high intermediate or high IPI score in the majority of patients. Similarly to what was first reported in a previous study,[6] we found that in addition to a subgroup of patients with a transformed DLBCL (7%), defined by a recognized previous history of indolent lymphoma, a significant fraction

FIGURE 2 Pattern of somatic mutations (with mutation rate ≥10%) in 54 patients with HCV-positive diffuse large B-cell lymphoma.

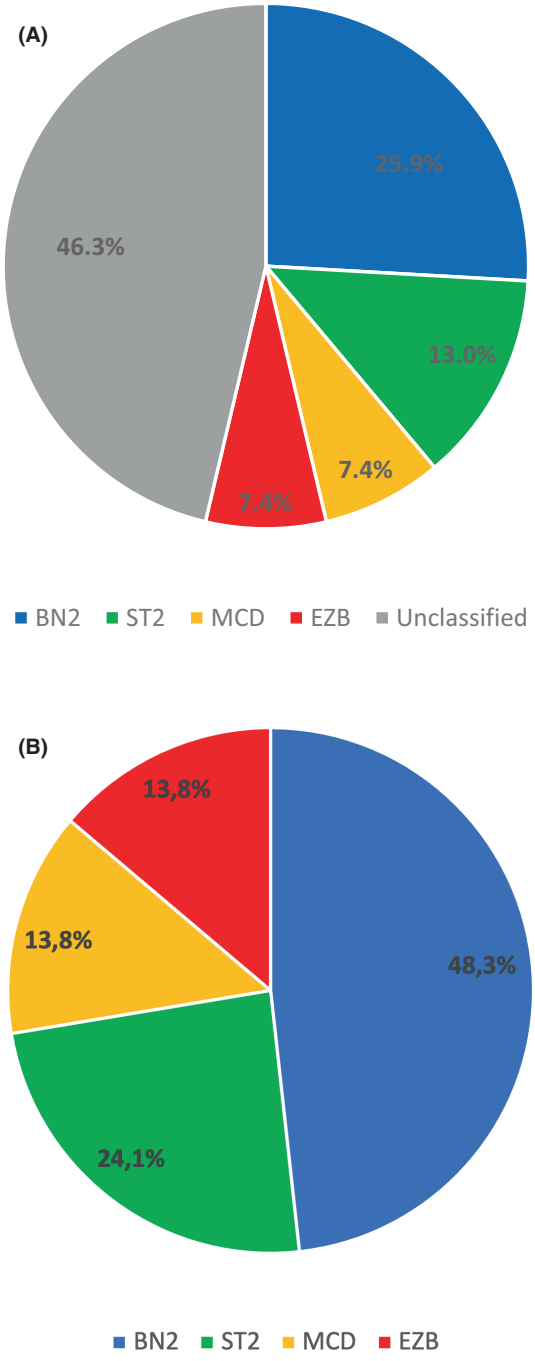

FIGURE 3 Cluster analysis by *LymphGen* tool, all patients (A) and classified patients (B).

of HCV-associated de novo DLBCL (15%) display histological features suggestive for an underlying MZL. These findings are in accordance with the most accepted model of HCV-driven lymphomagenesis, involving a chronic antigen stimulation-mediated process beginning from the achievement of B-cell clonality, progressing through the development of an indolent lymphoma (typically MZL) and finally acquiring a more aggressive behaviour (DLBCL).[1,6,30–32] However, at least in some cases of de novo DLBCL, a direct oncogenic effect exerted by the infection of lymphoma cells by HCV, as suggested by the detection of in situ NS3 viral protein expression by immunohistochemistry,[33] could not be ruled out.

Thus, our comprehensive cytogenetic, gene expression and mutational data may help to elucidate the molecular pathogenesis of HCV-associated DLBCL and, possibly, to suggest novel targeted therapeutic strategies.

With regards to chromosomal translocation, we confirmed that a low proportion of patients with HCV-associated DLBCL harbour *BCL2* rearrangements (4%) and, consequently, *BCL2/MYC* double hit profile (2%). These findings are in line with a previous case–control study that revealed the absence of *BCL2* translocation in 44 HCV-associated DLBCL cases, compared to 19% in HCV-negative ones.[19] Conversely, a large proportion of patients (51%) carried a *BCL6* rearrangement. Interestingly, this alteration has been reported in about 30% of cases of large cell variants of gastrointestinal extra-nodal MZL and large cell component of MALT/DLBCL composite lymphomas, suggesting *BCL6* as a candidate marker for transformation of MALT lymphoma.[34] *MYC* rearrangement was detected in 11% of patients, including 4% with *MYC/BCL6* rearrangements, in line with the literature data of HCV-negative DLBCL.[19,35]

Despite technical limitations in some samples due to the low extent or quality of tumour DNA extraction from archival tissue, in this study, we report a large series of HCV-associated DLBCL fully characterized in terms of COO as determined by GEP ($n = 38$). Similarly to a previously published series using the Affymetrix GeneChip platform,[36] we identified an equal proportion of ABC and GCB cases (42%); this is in contrast with another small cohort of elderly patients exhibiting a significantly lower proportion of ABC cases (18%) by Nanostring assay.[14] Notably, despite the small sample size, we showed a significantly worse survival among ABC cases with respect to GCB or Unclassified, similar to that demonstrated in HCV-negative DLBCL series treated with R-CHOP.[28] As the concordance with surrogate immunohistochemistry Hans algorithm was low (60%), our data emphasize the importance of COO determination through the Lymph2Cx assay also in the setting of HCV-associated DLBCL.

In this study, we described the mutational landscape of HCV-associated DLBCL. We confirmed the well-known high degree of heterogeneity and the high number of mutations typical of DLBCL. Interestingly, whereas some recurrently mutated genes (*KMT2D, PIM1, CREBBP, HIS1H1E, TP53, MYD88, TNAIFP3, NOTCH2*) were already reported in large published series of HCV-negative DLBCL,[15,18] we identified a set of genes who were mutated at higher frequency in our series of HCV-associated DLBCL (*SETD1B, RERE, FAS, BCL11A, TBL1XR1, SGK1, ZFP36L1, FBXO11, USH2A, ARID1A, KLF2*). Notably, a small independent series of HCV-associated DLBCL analysed by whole exome sequencing[22] identified a high rate of mutations in *ZFP36L1*, a gene encoding for a negative regulator of *NOTCH1* expression, and thus may represent a candidate gene involved in HCV-induced lymphomagenesis. Some of the genes

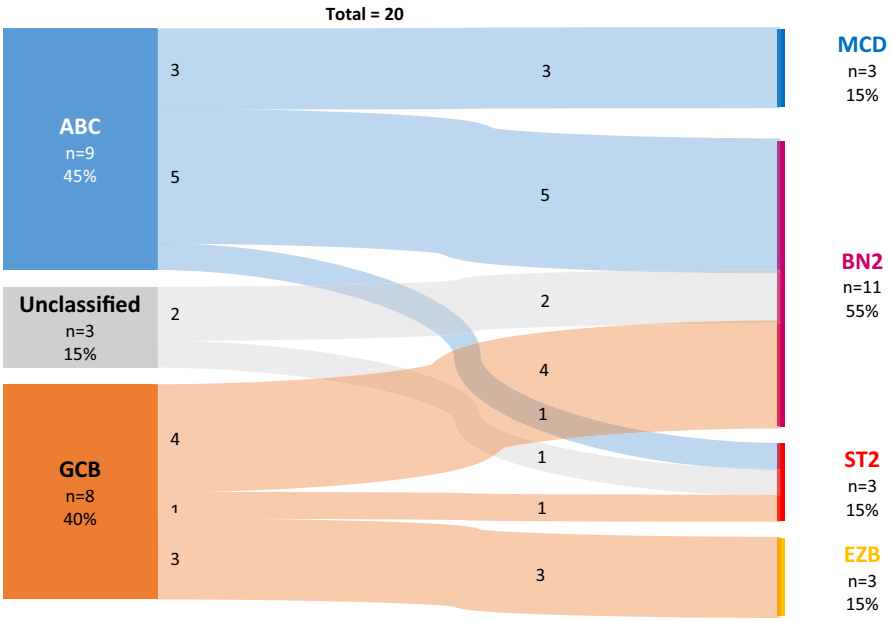

FIGURE 4 Correspondence between cell-of-origin by Nanostring and genetic subtypes by *LymphGen*.

described are typically mutated in MZL (*NOTCH2, KLF2, KMT2D, TNFAIP3*)[21] as well as in HCV-associated indolent lymphomas,[37] thus suggesting a possible multistep transformation pathway.

Overall, we found the most frequently involved pathways included those regulating epigenetics, apoptosis and BCR/NF-κB signalling. Interestingly, we confirmed the previous finding that the NOTCH pathway, one of the most frequently involved in MZL,[21,30,38] is also involved in approximately 25% of cases of HCV-associated DLBCL and that a mutation in these genes seems to be associated with a worse prognosis.[6] Surprisingly, we found that a mutation in genes involved in B-cell development (*PRDM1, BCL6, EBF1, IKZF3, ETS1*), observed in 25.9% of cases, was associated with better OS, although further investigation is required to confirm these findings.

Several studies in the last few years have developed new, albeit similar, genetic classifications of patients with HCV-negative DLBCL based on integrative multiplatform analysis of genetic aberrations, allowing for further clustering into homogeneous molecular subtypes.[15–18] We profiled our cohort using the web-based *LymphGen classifier*, based on the updated NCI classification.[17]

In line with the original report,[17] only 54% of our cases had a defined genetic subtype. Most cases clustered in the BN2 (48%) and ST2 (24%) subtypes, whereas 14% clustered in the MCD and EZB subtypes. Notably, the most represented cluster in our cohort was the BN2 subtype, whose genetic hallmarks reveal a potential pathogenic link to MZL. Indeed, the BN2 cluster, comprised of ABC, GCB and Unclassified cases,[15] typically exhibits *BCL6* structural alterations, which have been described as common in MZL developing an aggressive behaviour,[34] and is characterized by mutations deregulating two pathways supposed to be crucial for MZL development: the *NOTCH2*-mediated signalling (through *NOTCH2* activation or *SPEN* inactivation) and the BCR-dependent NF-κB pathway.[20] On this basis, the relatively higher prevalence of BN2 cluster observed in our cohort compared to the NCI series,[15,17] as well as to corresponding C1[16] or NOTCH cluster[18] in other classifications seems to strengthen the hypothesis that a consistent part of HCV-associated DLBCL cases may represent the transformed phase of an unrecognized MZL clone. Of ABC tumours in our series, the MCD subtype represented the second most frequent group, whose genetic signature is reminiscent of that observed in certain primary extra-nodal lymphomas, including central nervous system or skin,[17] the latter being relatively frequent in HCV-associated DLBCL.

Intriguingly, our Lymph2Cx- and *LymphGen*-generated data may have potential therapeutic implications in the specific setting of HCV-associated DLBCL, giving the preliminary finding of preferential activity of the BTK inhibitor ibrutinib in association with R-CHOP in younger patients with ABC DLBCL[39] and those with BN2, MCD[40] or N1 subtypes.[41] Moreover, the well-known activity of BTK inhibitors in relapsed or refractory MZL may suggest further exploration of these agents in HCV-associated DLBCL cases with genetically defined MZL derivation.[42–44]

We recognize the limitations of our data. First, due to the retrospective nature of the study and the rarity of HCV-associated DLBCL, patients were diagnosed across a long time interval and were not homogeneously treated; in particular, about half of the patients did not receive rituximab as part of first-line therapy. Moreover, some patients underwent HCV eradication therapy with DAA, which has been suggested to potentially improve long-term outcomes in this setting,[12–14,45] and thus may have influenced the prognosis of treated patients. Second, the relatively small

sample size and the failure to obtain GEP results due to suboptimal archival samples may have limited the prognostic results. Third, we did not evaluate somatic copy number alterations, including 17p deletion, precluding the identification of the A53 cluster. Finally, we were not able to confirm the somatic origin of mutations due to the lack of control germline samples.

In conclusion, our study comprehensively investigated the peculiar molecular landscape of HCV-associated DLBCL by integrating structural abnormalities by FISH, GEP by Lymph2Cx, mutational profile by NGS and genetic subtype allocation through *LymphGen*. We confirmed the scarcity of BCL2 translocations. We found the presence of the ABC signature in nearly 40% of cases and demonstrated its association with a poorer outcome. Moreover, we identified a set of genes (*SETD1B, RERE, BCL11A, TBL1XR1* and *ZFP36L1)* recurrently mutated at a higher frequency compared to HCV-negative DLBCL series and which may represent potential candidate genes involved in HCV-promoted lymphomagenesis. Finally, the involvement of NOTCH pathway signalling in about a quarter of cases and the prevalence of BN2 cluster (48% of cases) may suggest a preferential marginal zone origin in a consistent subgroup of HCV-associated DLBCL cases and thus potential implications for molecularly targeted therapies.

AUTHOR CONTRIBUTIONS

RS, MM, MP, FP and LA designed the research study. SZ, RR, DF, CV and LL performed the biological analyses. ML, SU, SF, FS, GR and MP performed the histological revision. VVF, FDP and ER analysed the data. RS, MM, CC, CZ, BB, MG, TL, ID, BM, AM, FC and MB collected clinical data. RS, MM, CC and LA interpreted the data and wrote the paper. All the authors revised and approved the final form of the manuscript.

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CONFLICT OF INTEREST STATEMENT

LA received honoraria from EUSA Pharma, Novartis and served on advisory boards for Roche, Janssen-Cilag, Verastem, Incyte, EUSA Pharma, Celgene/Bristol Myers Squibb, Kite/Gilead, ADC Therapeutics.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study was approved by the local ethics committee of the two participating institutions.

PATIENT CONSENT STATEMENT

Not applicable.

CLINICAL TRIAL REGISTRATION

Not applicable.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

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REFERENCES

1. Cacoub P, Saadoun D. Extrahepatic manifestations of chronic HCV infection. N Engl J Med. 2021;384(11):1038–52.
2. Besson C, Canioni D, Lepage E, Pol S, Morel P, Lederlin P, et al. Characteristics and outcome of diffuse large B-cell lymphoma in hepatitis C virus-positive patients in LNH 93 and LNH 98 Groupe d'Etude des Lymphomes de l'Adulte programs. J Clin Oncol. 2006;24(6):953–60.
3. Visco C, Arcaini L, Brusamolino E, Burcheri S, Ambrosetti A, Merli M, et al. Distinctive natural history in hepatitis C virus positive diffuse large B-cell lymphoma: analysis of 156 patients from northern Italy. Ann Oncol off J Eur Soc Med Oncol. 2006;17(9):1434–40.
4. Merli M, Visco C, Spina M, Luminari S, Ferretti VV, Gotti M, et al. Outcome prediction of diffuse large B-cell lymphomas associated with hepatitis C virus infection: a study on behalf of the Fondazione Italiana Linfomi. Haematologica. 2014;99(3):489–96.
5. Besson C, Noel N, Lancar R, Prevot S, Algarte-Genin M, Rosenthal E, et al. Hepatitis C virus or hepatitis B virus coinfection and lymphoma risk in people living with HIV. AIDS England. 2020;34(4):599–608.
6. Arcaini L, Rossi D, Lucioni M, Nicola M, Bruscaggin A, Fiaccadori V, et al. The notch pathway is recurrently mutated in diffuse large B-cell lymphoma associated with hepatitis c virus infection. Haematologica. 2015;100(2):246–52.
7. Pecoraro V, Banzi R, Cariani E, Chester J, Villa E, D'Amico R, et al. New direct-acting antivirals for the treatment of patients with hepatitis C virus infection: a systematic review of randomized controlled trials. J Clin Exp Hepatol. 2019;9(4):522–38.
8. Arcaini L, Besson C, Frigeni M, Fontaine H, Goldaniga M, Casato M, et al. Interferon-free antiviral treatment in B-cell lymphoproliferative disorders associated with hepatitis C virus infection. Blood. 2016;128(21):2527–32.
9. Frigeni M, Besson C, Visco C, Fontaine H, Goldaniga M, Visentini M, et al. Interferon-free compared to interferon-based

antiviral regimens as first-line therapy for B-cell lymphoproliferative disorders associated with hepatitis C virus infection. Leukemia. 2020;34(5):1462–6.
10. Peveling-Oberhag J, Arcaini L, Bankov K, Zeuzem S, Herrmann E. The anti-lymphoma activity of antiviral therapy in HCV-associated B-cell non-Hodgkin lymphomas: a meta-analysis. J Viral Hepat England. 2016;23(7):536–44.
11. Merli M, Rattotti S, Spina M, Re F, Motta M, Piazza F, et al. Direct-acting antivirals as primary treatment for hepatitis C virus–associated indolent non-Hodgkin lymphomas: the BArT study of the Fondazione Italiana Linfomi. J Clin Oncol. 2022;40(35):4060–70.
12. Merli M, Frigeni M, Alric L, Visco C, Besson C, Mannelli L, et al. Direct-acting antivirals in hepatitis C virus-associated diffuse large B-cell lymphomas. Oncologist. 2019;24(8):e720–e729.
13. Merli M, Defrancesco I, Visco C, Besson C, Di Rocco A, Arcari A, et al. Direct-acting antivirals in relapsed or refractory hepatitis C virus-associated diffuse large B-cell lymphoma. Leuk Lymphoma. 2020;61(9):2122–8.
14. Arcari A, Tabanelli V, Merli F, Marcheselli L, Merli M, Balzarotti M, et al. Biological features and outcome of diffuse large B-cell lymphoma associated with hepatitis C virus in elderly patients: results of the prospective "elderly project" by the Fondazione Italiana Linfomi. Br J Haematol England. 2023;201(4):653–62.
15. Schmitz R, Wright GW, Huang DW, Johnson CA, Phelan JD, Wang JQ, et al. Genetics and pathogenesis of diffuse large B-cell lymphoma. N Engl J Med. 2018;378(15):1396–407.
16. Chapuy B, Stewart C, Dunford AJ, Kim J, Kamburov A, Redd RA, et al. Molecular subtypes of diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes. Nat Med. 2018;24(5):679–90.
17. Wright GW, Huang DW, Phelan JD, Coulibaly ZA, Roulland S, Young RM, et al. A probabilistic classification tool for genetic subtypes of diffuse large B cell lymphoma with therapeutic implications. Cancer Cell. 2020;37(4):551–68. e14.
18. Lacy SE, Barrans SL, Beer PA, Painter D, Smith AG, Roman E, et al. Targeted sequencing in DLBCL, molecular subtypes, and outcomes: a Haematological malignancy research network report. Blood. 2020;135(20):1759–71.
19. Visco C, Wang J, Tisi MC, Deng L, D'Amore ESG, Tzankov A, et al. Hepatitis C virus positive diffuse large B-cell lymphomas have distinct molecular features and lack BCL2 translocations. Br J Cancer. 2017;117(11):1685–8.
20. Rossi D, Trifonov V, Fangazio M, Bruscaggin A, Rasi S, Spina V, et al. The coding genome of splenic marginal zone lymphoma: activation of NOTCH2 and other pathways regulating marginal zone development. J Exp Med. 2012;209(9):1537–51.
21. Bonfiglio F, Bruscaggin A, Guidetti F, Terzi di Bergamo L, Faderl M, Spina V, et al. Genetic and phenotypic attributes of splenic marginal zone lymphoma. Blood. 2022;139(5):732–47.
22. Armand M, Degaud M, Tesson B, Laurent C, Vavasseur M, Parisot M, et al. Exploring the genetic landscape of HCV-related B-cell lymphomas using whole exome sequencing. Leukemia. 2023;37(6):1388–91.
23. Alaggio R, Amador C, Anagnostopoulos I, Attygalle AD, de Araujo IBO, Berti E, et al. The 5th edition of the World Health Organization classification of Haematolymphoid Tumours: lymphoid neoplasms. Leukemia. 2022;36(7):1720–48.
24. Cheson BD, Fisher RI, Barrington SF, Cavalli F, Schwartz LH, Zucca E, et al. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. J Clin Oncol. 2014;32(27):3059–67.
25. International Non-Hodgkin's Lymphoma Prognostic Factors Project. A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med. 1993;329(14):987–94.
26. Sehn LH, Berry B, Chhanabhai M, Fitzgerald C, Gill K, Hoskins P, et al. The revised international prognostic index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP. Blood. 2007;109(5):1857–61.
27. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004;103(1):275–82.
28. Scott DW, Wright GW, Williams PM, Lih C-JJ, Walsh W, Jaffe ES, et al. Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. Blood. 2014;123(8):1214–7.
29. Cheson BD, Pfistner B, Juweid ME, Gascoyne RD, Specht L, Horning SJ, et al. Revised response criteria for malignant lymphoma. J Clin Oncol. 2007;25(5):579–86.
30. Rossi D, Bertoni F, Zucca E. Marginal-zone lymphomas. N Engl J Med. 2022;386(6):568–81.
31. Merli M, Arcaini L. Management of marginal zone lymphomas. Hematology. 2022;2022(1):676–87.
32. Merli M, Passamonti F, Arcaini L. Deciphering molecular complexity of HCV-associated lymphoproliferation. Br J Haematol England. 2023;203(2):154–7.
33. Canioni D, Michot J-M, Rabiega P, Molina TJ, Charlotte F, Lazure T, et al. In situ hepatitis C NS3 protein detection is associated with high grade features in hepatitis C-associated B-cell non-Hodgkin lymphomas. PloS One. 2016;11(6):e0156384.
34. Flossbach L, Antoneag E, Buck M, Siebert R, Mattfeldt T, Möller P, et al. BCL6 gene rearrangement and protein expression are associated with large cell presentation of extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue. Int J Cancer. 2011;129(1):70–7.
35. Rosenwald A, Bens S, Advani R, Barrans S, Copie-Bergman C, Elsensohn M-H, et al. Prognostic significance of MYC rearrangement and translocation partner in diffuse large B-cell lymphoma: a study by the Lunenburg lymphoma biomarker consortium. J Clin Oncol Off J Am Soc Clin Oncol. 2019;37(35):3359–68.
36. Xu-Monette ZY, Dabaja BS, Wang X, Tu M, Manyam GC, Tzankov A, et al. Clinical features, tumor biology, and prognosis associated with MYC rearrangement and Myc overexpression in diffuse large B-cell lymphoma patients treated with rituximab-CHOP. Mod Pathol. 2015;28(12):1555–73.
37. Defrancesco I, Visentini M, Zibellini S, Minafò YA, Rattotti S, Ferretti VV, et al. Mutational and immunogenetic landscape of HCV-associated B-cell lymphoproliferative disorders. Am J Hematol. 2021;96:E210–E214.
38. Parry M, Rose-Zerilli MJJ, Ljungström V, Gibson J, Wang J, Walewska R, et al. Genetics and prognostication in splenic marginal zone lymphoma: revelations from deep sequencing. Clin Cancer Res. 2015;21(18):4174–83.
39. Younes A, Sehn LH, Johnson P, Zinzani PL, Hong X, Zhu J, et al. Randomized phase III trial of ibrutinib and rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone in non-germinal center B-cell diffuse large B-cell lymphoma. J Clin Oncol Off J Am Soc Clin Oncol. 2019;37(15):1285–95.
40. Zhang M-C, Tian S, Fu D, Wang L, Cheng S, Yi H-M, et al. Genetic subtype-guided immunochemotherapy in diffuse large B cell lymphoma: the randomized GUIDANCE-01 trial. Cancer Cell. 2023;41(10):1705–1716.e5.
41. Wilson WH, Wright GW, Huang DW, Hodkinson B, Balasubramanian S, Fan Y, et al. Effect of ibrutinib with R-CHOP chemotherapy in genetic subtypes of DLBCL. Cancer Cell. 2021;39(12):1643–1653.e3.
42. Noy A, de Vos S, Coleman M, Martin P, Flowers CR, Thieblemont C, et al. Durable ibrutinib responses in relapsed/refractory marginal zone lymphoma: long-term follow-up and biomarker analysis. Blood Adv. 2020;4(22):5773–84.
43. Opat S, Tedeschi A, Linton K, McKay P, Hu B, Chan H, et al. The MAGNOLIA trial: Zanubrutinib, a next-generation Bruton tyrosine kinase inhibitor, demonstrates safety and efficacy in relapsed/refractory marginal zone lymphoma. Clin Cancer Res. 2021;27(23):6323–32.
44. Matasar MJ, Luminari S, Barr PM, Barta SK, Danilov AV, Hill BT, et al. Follicular lymphoma: recent and emerging therapies, treatment strategies, and remaining unmet needs. Oncologist. 2019;24(11):e1236–e1250.

45. Merli M, Frigeni M, Gotti M, Grossi P, Bruno R, Passamonti F, et al. Direct-acting antivirals during or after immunochemotherapy in hepatitis C virus-positive diffuse large B-cell lymphomas. Hepatology. 2017;66(4):1341–3.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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