**c-MYC and p53 expression highlight starry-sky pattern as a favourable prognostic feature in R-CHOP-treated diffuse large B-cell lymphoma**

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**Abstract**

Diffuse large B-cell lymphoma (DLBCL) is a clinically heterogeneous entity, in which the first-line treatment currently consists of an immuno–chemotherapy regimen (R-CHOP). However, around 30% of patients will not respond or will relapse. Overexpression of c-MYC or p53 is frequently found in DLBCL, but an association with prognosis remains controversial, as for other biomarkers previously linked with DLBCL aggressivity (CD5, CD23, or BCL2). The aim of this study was to explore the expression of these biomarkers and their correlation with outcome, clinical, or pathological features in a DLBCL cohort. Immunohistochemical (c-MYC, p53, BCL2, CD5, and CD23), morphological (‘starry-sky’ pattern [SSP]), targeted gene panel sequencing by next-generation sequencing (NGS), and fluorescence in situ hybridisation analyses were performed on tissue microarray blocks for a retrospective cohort of 94 R-CHOP-treated de novo DLBCL. In univariate analyses, p53 overexpression (p53high) was associated with unfavourable outcome (p = 0.04) and with c-MYC overexpression (p = 0.01), whereas c-MYC overexpression was linked with an SSP (p = 0.004), but only tended towards an inferior prognosis (p = 0.06). Presence of a starry-sky morphology was found to be correlated with better survival in p53high DLBCL (p = 0.03) and/or c-MYC-positive DLBCL (p = 0.002). Furthermore, NGS data revealed that these three variables were associated with somatic mutations (PIM1, TNFRSF14, FOXO1, and B2M) involved in B-cell proliferation, survival, metabolism, and immune signalling. Taken together, these results show that the SSP pattern seems to be a protective factor in high-risk DLBCL subgroups and highlight cell death as a built-in failsafe mechanism to control tumour growth.

**Keywords:** DLBCL; R-CHOP; p53; prognostic; starry-sky; c-MYC; protective; factor

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is a clinically heterogeneous entity, accounting for around 30% of all lymphomas [1] and is the most common haematological malignancy in Western countries [2,3]. DLBCL is also among the most aggressive lymphoid neoplasms, with a 5-year overall survival (OS) of around 50% [4]. Currently, a significant proportion of patients will still not have a primary response or will relapse with the standard of care, R-CHOP (rituximab, a humanised monoclonal CD20 antibody plus cyclophosphamide, doxorubicin hydrochloride, vincristine sulphate, and prednisolone). Although outcomes in DLBCL have improved with this immunotherapy [5,6], around 10–15% of patients exhibit primary refractory disease and an additional 20–25% relapse, usually within the first 2 years [5], highlighting the limits of R-CHOP and the need for new predictive biomarkers that could guide individualised risk-adapted therapy [7]. Currently, the only robust prognostic factor is the International Prognostic Index (IPI) score [8], revised in the era of R-CHOP [9] (r-IPI), which segregates patients into different outcome groups.

Advances in molecular genetics have confirmed the biological heterogeneity of DLBCL [10,11] through the identification of several alterations leading to oncogene activation and modulation of tumour suppressor activity [12,13]. Patterns of gene expression reminiscent of germinal centre B cell (GCB) group and activated B cell (ABC) group allowed the definition of the ‘cell of origin’ DLBCL classification, the GCB subtype being associated with better prognosis [10]. New entities have also been described such as high-grade B-cell lymphoma (HGBL), encompassing HGBL double-hit (DHL)/triple-hit lymphomas (THL) and HGBL, NOS (not otherwise specified). HGBL DHL or THL are defined by MYC and BCL2 and/or BCL6 rearrangements, whereas HGBL, NOS presents a blastoid morphology or an intermediate aspect between DLBCL, NOS, and Burkitt lymphomas and lacking DHL or THL [14,15]. More recently, different molecular DLBCL subgroups associated with outcomes have been uncovered based on mutational profiling [16,17]. However, although these investigations have shed light on the biological heterogeneity of DLBCL and revealed new potential therapeutic targets, they failed to precisely discriminate very high-risk DLBCL patients.

Immunohistochemistry (IHC) is routinely used to classify DLBCL as GCB or non-GCB using Hans’ algorithm [18] and to evaluate expression of several biomarkers associated with DLBCL aggressivity, including c-MYC, BCL2, p53, CD5, or CD23. Of note, c-MYC, BCL2, and BCL6 overexpression may occur independent of MYC, BCL2, or BCL6 gene rearrangement [19].

MYC is a proto-oncogene encoding the transcription factor c-MYC controlling cellular processes such as cell cycle and cell growth [20]. Its oncogenic activation has been described in Burkitt lymphomas and in 10% of DLBCL [19,21]. It has been proven that MYC rearrangement has an adverse effect on survival in R-CHOP-treated DLBCL, especially if the partner is an immunoglobulin (Ig) gene [22]. Although c-MYC protein expression is found in 30–50% of DLBCL (according to standard cut-off values) [23], its survival significance is still controversial even if it seems to correlate with poorer outcomes in the rituximab era [19,23–25]. BCL2 is an anti-apoptotic gene encoding the BCL2 protein which is overexpressed in approximately 50% of DLBCL [26] and has been a well-recognised prognostic factor in the pre-rituximab era [27,28]. Nowadays, the addition of rituximab can mitigate the adverse effects of BCL2 expression, especially in the ABC subtype [29,30]. Furthermore, double-expressor lymphoma (DEL), defined by co-expression of c-MYC and BCL2, is also associated with a poor prognostic effect but with a stronger negative impact than BCL2 or c-MYC overexpression taken individually [14,31]. The protein p53 (or TP53) is encoded by the gene TP53 (also known as p53), a well-known tumour suppressor mediating several crucial cell functions such as cell cycle arrest, apoptosis, or DNA reparation [32]. Thus, p53 mutations (gain or loss of function) are found in many cancers, including in 20% of DLBCL [13], and confer inferior survival [33,34]. Protein overexpression is more common (30–40% of DLBCL are p53-positive, depending on the positivity threshold) and is also predictive of poorer prognosis in R-CHOP-treated patients [33,35].

Moreover, some studies showed that p53 overexpression enhances c-MYC-related adverse effects in DLBCL, identifying a high risk of relapse subgroup of patients co-expressing c-MYC and p53 [34,36]. Other IHC biomarkers can also be used, such as CD5 and CD23, which are positive in 5–10% of DLBCL [37,38] but their significance still remains controversial. On one hand, CD23 is expressed on naïve B cells and follicular dendritic cells and its overexpression has been associated with unfavourable outcomes in DLBCL [38,39]. On the other hand, CD5 is expressed on normal T cells but as it is also positive in other lymphoid malignancies (chronic lymphocytic leukaemia and Mantle cell lymphoma), further analyses (Cyclin D1 and SOX11) are needed to exclude these differential diagnoses. De novo CD5-positive DLBCL seems to be associated with adverse clinical and...
biological features [40] and with poorer OS and progression-free survival (PFS) after chemotherapy compared to CD5-negative DLBCL [37,41].

Finally, some DLBCL have been shown to exhibit a morphological ‘starry-sky’ pattern (SSP). SSP, also found in Burkitt lymphoma, is defined by the presence of tingible-body macrophages (appearing as ‘stars’ because they contain cellular debris within their cytoplasm) intermingled with a ‘sky’ of tumour cells, and is a reflection of a high mitotic rate and a high degree of apoptosis [42]. Macrophage proliferation in SSP has been described as promoted by lymphoma cell apoptosis [43].

The aim of the present study was to explore, in a retrospective cohort of R-CHOP-treated DLBCL, the correlation between SSP and expression of several biomarkers (c-MYC, p53, BCL2, CD5, and CD23) with outcome, clinical, pathological, and molecular features.

Materials and methods

Patient selection

Ninety-four patients were included in a monocentric retrospective study in Grenoble, France (Centre Hospitalier Universitaire Grenoble Alpes and Institut Daniel Hollard, Department of Hematology) from January 2011 to December 2018. All patients had signed an institutional consent form (reference AC-2014-2094 no. 9). Each case was reviewed to ensure compliance with the following inclusion criteria. DLBCL tumours had to be de novo, and all patients with a history of other lymphoma and relapses were excluded. Primitive cerebral DLBCL, DLBCL leg-type, HGBL, and primary mediastinal B-cell lymphoma were discarded because of their particular pathological and biological presentations. Treatment had to be R-CHOP in the first line (at least 3 cycles). Tissues had to be formalin-fixed paraffin-embedded Alcohol, Formalin, and Acetic acid (AFA) fixation was excluded to avoid IHC bias) and enough residual material had to be available to perform tissue microarray (TMA) construction (needle biopsies were not included). Biological and clinical data were collected for each patient, including gender, age, IPI score, Ann Arbor stage, serum lactate dehydrogenase level as well as detailed treatment and long-term survival data. DLBCL were classified according to the WHO classification with the GCB or non-GCB status using Hans’ algorithm. Finally, a morphological review was conducted to search for an SSP for each case.

TMA construction

Five TMA blocks were built as described previously [44]. TMAs were composed of four 0.6 mm cores from each patient’s samples and non-tumour samples were also added as internal positive controls (normal appendix and testis).

Morphological analysis

TMA tissue spots were reviewed by a morphological analysis (haematoxylin–eosin–saffron-stained section) to exclude those with an unrepresentative aspect (fibrotic or necrotic changes) and to ensure that every case showed a morphology compatible with a DLBCL, NOS.

Immunohistochemistry

IHC analysis was performed on 4-μm thick sections cut from TMA blocks. A fully automated (baking, deparaffinisation, antigen retrieval, IHC, and counterstaining) BenchMark ULTRA (Roche Diagnostics, Meylan, France) was used for all antibodies tested. Nine antibodies were used for each TMA: c-MYC, BCL2, p53, CD5, CD23, MUM-1, Cyclin D1, SOX11, and Ki67 (see supplementary material, Table S1) and a routinely used procedure was followed. For c-MYC IHC, the UltraView™ Universal DAB Detection Kit (Ventana Medical Systems Inc., Tucson, AZ, USA) was used to increase the staining intensity. The cut-off for c-MYC positivity was more than 40% of tumour cells showing immunoreactivity, whereas thresholds for BCL2, p53, CD5, and CD23 were more than 50%, in accordance with previously established cut-offs [31,33,37,38]. For Ki67, results were rendered semi-quantitatively on a scale from 1 to 4+, according to the percentage of positive cells (1+ [1–25%], 2+ [26–50%], 3+ [51–75%], and 4+ [76–100%]) [18]. IHC staining was scored ‘blindly’ by two pathologists (AB and HS) and discordant cases were subjected to a joint proof reading.

Antibodies against MUM1, Cyclin D1, and SOX11 were used to exclude DLBCL with IRF4 rearrangement and differential diagnoses for all de novo CD5-positive DLBCL.

EBER in situ hybridisation

To identify EBV-positive DLCBL, NOS, we performed Epstein-Barr virus-encoded small RNA (EBER) in situ hybridisation (ISH) on deparaffinised 4-μm thick sections from TMA blocks using a BenchMark ULTRA automated tissue stainer, a fluorescein-conjugated EBER oligonucleotide probe (Roche Diagnostics), and an INFORM iView Blue ISH detection kit (Roche Diagnostics).
Diagnoses. Slides were counterstained using Red Counterstain II (Roche Diagnostics). Slides were assessed by two pathologists (AB and HS) and 80% of EBER-positive tumour cells was the cut-off applied to consider a DLBCL EBV-positive [1].

Fluorescence in situ hybridisation
To be sure of the absence of HGBL DHL/THL in our cohort, interphase fluorescence in situ hybridisation (FISH) was performed using 4-μm thick TMA sections. All samples were tested using MYC, BCL2, and BCL6 break-apart FISH using the ZytoLight® SPEC MYC Dual Color Break Apart Probe, the ZytoLight® SPEC BCL2 Dual Color Break Apart Probe, and the ZytoLight® SPEC BCL6 Dual Color Break Apart Probe (ZytoVision, Bremerhaven, Germany), respectively. In addition, a Vyssis LSI IGH/MYC/CEP 8 Tri-Color Dual Fusion Probe Kit (Abbott Laboratories, Abbott Park, IL, USA) was used to determine if IGH was the partner gene of MYC rearrangement. At least 100 tumour nuclei were analysed for each sample. Nuclei were counterstained with DAPI/Vectashield® (Vector Laboratories, Burlingame, CA, USA) and were analysed using a Leica CytoVision GSL10 FISH fluorescence capture system® (Leica, Nanterre, France) under a ×63 oil immersion objective. Signals were counted using the CytoVision imaging system® (Leica). A cut-off value of 10% of the cells showing rearrangement was selected to confirm rearrangement for a specimen.

Targeted NGS
Four 0.6-mm FFPE core biopsies were used per case at the same time as TMAs were built. DNA was extracted using a QIAsymphony DSP DNA Mini Kit (Qiagen, Hilden, Germany). DNA quality was assessed by TepeseqStation analysis and quantified for next-generation sequencing (NGS) by fluorometry (ThermoFisher Scientific, Waltham, MA, USA). Targeted sequencing was performed by a capture approach covering 51 genes that present mutations in DLBCL, follicular lymphoma, and chronic lymphocytic leukaemia [45]. Libraries were prepared by standard procedures using 50 ng of DNA and a KAPA HyperPlus kit (Roche Diagnostics). Libraries were purified, quantified, and barcoded before being sequenced using a NextSeq 500/550 Mid Output Kit V2 (300 cycles) kit and a NextSeq 550 Illumina sequencer (Illumina, San Diego, CA, USA). Somatic mutation calling was performed using an in-house sequence alignment, variant caller, and annotation pipeline. Somatic mutations that were present at >5% variant allele frequency and that affected protein coding regions (non-synonymous) or exon splice site junctions were retained for analysis. All somatic variants were reviewed manually for specificity and pathogenicity.
Statistics
OS and PFS were assessed by Kaplan–Meier analyses and Cox regression. The starting point for time-to-event analysis was the date of the pathological diagnosis. Observational intervals of patients without any event at the time of the last follow-up or at 7.5 years after diagnosis were censored. The proportional hazards assumption was checked on the basis of Schoenfeld residuals after Cox model fitting. The concordance probability, which is defined as the probability that predictions and outcomes are concordant, was provided by Harrell's C and Gönen and Heller's concordance coefficients.

All statistical analyses were performed using Stata 16.1 (Stata Corporation, College Station, TX, USA) and R statistical software 3.5.0 (The R Foundation for Statistical Computing, Vienna, Austria). All statistical tests were two-sided, and the level for significance was set at $p < 0.05$.

Results

Cohort clinical characteristics
A total of 94 patients were included; the main clinical characteristics are presented in Table 1. In brief, within this cohort, the gender distribution was balanced with a slight predominance of male patients (56.3%) and a median age at diagnosis of 67 years. The average time...
of long-term follow-up was over 3 years (41 months). Around half of the patients presented an advanced stage DLBCL (Ann Arbor stage IV in 45.7% and a 3–5 IPI score in 49.0%).

Cohort pathological features

The main pathological characteristics are summarised in Table 2. Following morphological assessment, all DLBCL showed a typical morphology. No case of HGBL, NOS was identified. SSP was seen in 29.8% of cases (Figure 1A). Using Hans’ algorithm, more non-GCB DLBCL were observed than GCB (63.8 versus 36.2%). Regarding IHC analyses, p53 was found positive (p53high) in 40.0% of DLBCL (Figure 1B), c-MYC was expressed in around one-third of cases (30/94, Figure 1C), and BCL2 was overexpressed in 57 patients (60.6%, Figure 1D). Patients with DEL accounted for nearly one-quarter of this cohort (21.3%). CD5 expression was observed in 8 cases (8.5%, Figure 1E) and 10 patients were CD23 positive (10.6%, Figure 1F). Of note, all CD5-positive DLBCL were negative for Cyclin D1 and SOX11 and no MUM1 overexpression compatible with an IRF4 rearrangement was observed. Using Ki67 staining, more than half of the cases showed a 4+ proliferation index (52.1%). Only one patient provided a positive EBER ISH result.

FISH analyses revealed five patients had MYC rearrangement (Figure 2A,B), including three cases of MYC-IgH rearrangement (Figure 2C,D), and these five patients with MYC rearrangement were c-MYC-positive by IHC. We did not find any HGBL DHL/THL in our cohort.

Cohort validation

As expected, this cohort shows the inferior outcome associated with the non-GCB subtype compared to GCB DLBCL (p = 0.005, see supplementary material, Figure S1A). Prognostic impact of the R-IPI class was also confirmed with a better outcome for patients with IPI = 0 compared to patients with IPI = 1–2 or
IPI = 3–5 \( (p = 0.0008, \text{see supplementary material, Figure S1B}) \).

Impact of c-MYC-positive DLBCL on clinical features and outcomes

Overexpression of c-MYC was significantly correlated with IPI score higher than 2 (odds ratio [OR] = 3.4, \( p = 0.01 \)), with SSP (OR = 3.9, \( p = 0.004 \)), and with 4+ proliferation index (OR = 7.4, \( p = 0.0005 \)). DLBCL with c-MYC overexpression tended to have a worse prognosis (\( p = 0.06 \), Figure 3A), whereas SSP did not impact survival in our cohort (\( p = 0.38 \), see supplementary material, Figure S2A). However, c-MYC-positive DLBCL without SSP defined a subgroup of patients with inferior survival compared to those showing SSP (for OS, \( p = 0.002 \), Figure 3B, and for PFS, \( p = 0.001 \), see supplementary material, Figure S2B). In c-MYC-negative DLBCL subgroup, SSP did not impact survival.

Impact of p53\text{high} DLBCL on clinical features and outcomes

In univariate survival analysis, p53\text{high} DLBCL presented poorer outcome (\( p = 0.04 \), Figure 3C). This p53 prognostic impact was even stronger when DLBCL did not present SSP (\( p = 0.03 \), Figure 3D). Moreover, in the present study, p53 overexpression was linked with c-MYC overexpression (OR = 3.4, \( p = 0.01 \)) but not with SSP.

SSP DLBCL morphology was also able to segregate two prognostic groups within p53\text{high} and c-MYC-positive DLBCL (\( p = 0.004 \), see supplementary material, Figure S2E). As for c-MYC, SSP was not correlated with survival in p53\text{low} DLBCL subgroup.

Impact of other biomarkers on clinical features and outcomes

None of the other biomarkers tested showed an association with outcome (DEL, BCL2-positive DLBCL, CD23-positive DLBCL, CD5-positive DLBCL, and...
DLBCL with a 4+ proliferation index). We noted that SSP was more frequently found in DEL (OR = 4.1, \( p = 0.01 \)) and 4+ proliferation index DLBCL (OR = 3.2, \( p = 0.02 \)). As with c-MYC-positive and p53\(^{\text{high}} \) DLBCL, the presence of SSP defined a subgroup of patients with better survival in DEL.

Figure 4. NGS analysis of 48 DLBCL using a 51 gene lymphoma panel.
Correlation with NGS data

NGS lymphopanel sequencing was performed for 48 DLBCL and correlated with p53 and c-MYC expression and DLBCL morphology (Figure 4). The most frequently mutated gene was TP53 (16/38, 42.1%) and mutation profiles were broadly in line with GCB versus ABC patterns. c-MYC overexpression seemed enriched for B2M and FOXO1 mutations (6 of 18 cases in c-MYC-positive DLBCL compared to 1 of 30 cases in c-MYC-negative DLBCL, OR = 8.2, p = 0.004).

SSP DLBCL was not significantly associated with any specific mutational profile. Nevertheless, some degree of correlation was observed between DLBCL without SSP and TNFRSF14 mutations, without reaching significance threshold (9 of 32 cases, OR = 3.1, p = 0.07). TNFRSF14 mutations were significantly linked with p53high DLBCL without SSP (5 of 14 cases, OR = 3.7, p = 0.05).

Finally, in the p53high subgroup, 5 of 8 cases with SSP DLBCL showed PIM1 mutations compared to 1 of 14 DLBCL without this pattern (OR = 7.8, p = 0.005).

Discussion

The aim of this study was to assess the impact of different biomarkers on survival in R-CHOP-treated DLBCL. To achieve this evaluation, we established a retrospective cohort representative of de novo DLBCL in term of baseline characteristics, including inferior outcomes for patients with a high IPI score and a non-GCB subtype, both known as robust prognostic factors in DLBCL [7,18].

c-MYC protein overexpression was found in one-third of the cases, in agreement with previously reported data [23,24]. In univariate analysis, this overexpression showed only a weak trend with survival but some studies have drawn similar conclusions [31,46]. The prognostic impact of c-MYC expression still remains controversial in DLBCL. This could be explained by the absence of consensus in IHC cut-off positivity but also by a lack of treatment homogeneity in the cohort resulting in a substantial percentage of patients treated not only by R-CHOP. Furthermore, supporting previous reports, c-MYC-positive DLBCL in this study showed a strong interaction with a high IPI score [24,25] and a high proliferation index [19,31].

c-MYC overexpression was significantly associated with the presence of SSP, which is defined here by the presence, even focal, of tingible-body macrophages intermingled with tumour cells. In our work, to counterbalance the potential interpretation bias in evaluating the presence of SSP, we took four DLBCL samples per case to maximise tumour representativeness on the TMAs. In the literature, a correlation of c-MYC/SSP was once described but this was only between SSP and c-MYC gene rearrangement [47]. Moreover, a statistical analysis confirmed that these two factors could stratify DLBCL patients into prognostically relevant subgroups. These data strongly suggest that the adverse effect of c-MYC expression on survival could be outbalanced by SSP, which is correlated with a high apoptotic rate in the tumour [43], indicating that a high degree of apoptosis is a protective factor in c-MYC-driven DLBCL. This is consistent with findings in experimental oncogenesis where loss of pro-apoptotic signalling via p53 favours outgrowth of c-MYC overexpressing B-cell tumour clones [48]. Our data suggest that immune checkpoint escape (B2M) or FOXO1 signalling might also confer selective advantages for outgrowth of c-MYC overexpressing B-cell clones. Indeed, FOXO1 loss-of-function mutations are reported to confer poor prognosis in low-risk IPI DLBCL [49]. The association with MYC expression has not been previously reported.

Regarding p53 expression, in accordance with previous works [34], the p53high DLBCL subgroup was associated with c-MYC overexpression and with unfavourable outcomes. Moreover, the prognostic impact of p53 expression was even stronger in combination with SSP. As for c-MYC, p53high DLBCL without SSP had worse outcomes. The same applies for both c-MYC-positive and p53high cases confirming apoptosis as a protective factor in both p53 and c-MYC-driven lymphomas. This could be related to the well-known role of p53 in cell survival [50] and by upregulation of p53 expression by c-MYC [51].

Overall, SSP has a clear protective impact on p53high and c-MYC-positive DLBCL but the link between these three variables could be strengthened by a complementary multivariate analysis on a larger cohort.

In this cohort, NGS data revealed that p53, c-MYC, and SSP were linked with a specific mutational profile including several cell survival genes, such as FOXO1, B2M, TNFRSF14, and PIM1.

c-MYC-positive DLBCL presented a significant enrichment for FOXO1 and B2M mutations.
is known to play a key role in homeostatic control of B-cell survival and proliferation in particular in the germinal centre reaction where MYC and p53 are also key and in metabolic signalling [49,52,53]. Mutations in FOXO1, therefore considered as tumour suppressors, are also associated with decreased OS in c-MYC-driven DLBCL [48,49,53]. However, B2M mutations completely blocked the B2M-mediated apoptosis programme [54], representing another independent pathway by which MYC-driven DLBCL could escape apoptosis.

Interestingly, we found that p53\textsuperscript{high} DLBCL without SSP had a higher frequency of TNFRSF14 mutations which have been associated with unfavourable prognosis in follicular lymphomas [55]. In physiological conditions, the TNFRSF14 receptor encoded by this gene has a key role in cell survival and homeostasis of T and B cells, while in lymphoma, loss of function has been implicated in shaping the tumour microenvironment [54,56]. It is considered as a tumour suppressor gene, inducing apoptosis and immune response against tumour cells [56]. Conversely, as well as driving cell autonomous proliferation, TNFRSF14 mutation seems to create a tumour-supportive microenvironment and promote tumourigenesis [57]. Furthermore, it was shown that restoration of TNFRSF14 suppresses tumour growth in xenograft models, thus highlighting this receptor as an interesting therapeutic target in lymphoma [57]. TNFRSF14 mutation frequency in p53\textsuperscript{high} DLBCL without SSP suggests that this gene is a major player in the anti-tumour immune response and apoptosis in p53-driven DLBCL.

Lastly, PIM1 mutations were significantly linked with p53\textsuperscript{high} DLBCL with SSP. This gene has a well-characterised role in cell survival and is seen as a proto-oncogene acting synergistically with genes encoding proteins involved in cell death, especially p53 [58,59]. PIM1 oncogenic effects are effective only if tumour cells can bypass p53-dependent apoptosis by inhibiting p53 expression [59]. Oncogenic PIM1 also cooperates with MYC signalling to enhance oncogenic signalling in lymphoma which is known to select for p53 loss-of-function events. Thus, we make the assumption that, by maintaining p53 overexpression which induces high cell death level (with SSP as a consequence), cells are protecting themselves from PIM1 oncogenic mutations.

DEL accounted for 22.7% of this cohort, a ratio in agreement with other studies [14,31]. Here, DEL was associated with SSP, which had not previously been described. DEL without SSP presented poorer outcomes than DEL with this morphology, strengthening the association between activation of apoptotic pathways and improved survival. 60.8% of DLBCL cases were BCL2-positive [29,31] but they were not associated with inferior outcomes, consistent with a large number of studies on R-CHOP-treated DLBCL [29,30]. These data strongly suggest that the adverse effect of BCL2 overexpression on DLBCL outcome could be mitigated by the addition of rituximab as previously reported [29,30].

A high proliferation index (Ki67 = 4+) was found in more than half of the patients but was not correlated with outcome in our cohort. The question of this parameter as an independent and robust prognostic factor in R-CHOP-treated DLBCL is still controversial and a topic of debate. Some studies have shown that it predicts a worse survival in this lymphoma [60] but other works have concluded the opposite [31,61].

Finally, we found expected proportions of de novo CD5-positive (9 cases) and CD23-positive DLBCL (10 cases) [37,38] but they were not associated with outcomes. However, these results should be taken with caution because of the limited number of positive cases.

In conclusion, on a representative cohort of DLBCL uniformly treated by R-CHOP as the first-line therapy, only p53 overexpression was associated with poorer outcomes in univariate analysis. However, c-MYC and p53 overexpression was associated with improved survival when combined with SSP, which is a morphological pattern found in lymphomas with a high rate of apoptosis [43]. Thus, persistence of a high rate of apoptosis seems to have a protective impact when c-MYC and/or p53 are overexpressed. Although requiring confirmation in a larger cohort, this pro-apoptotic environment, controlled by several key genes including MYC, p53, FOXO1, B2M, TNFRSF14, and PIM1, could therefore indicate a key tumour suppressor pathway. Taken together, these results show an apoptosis-mediated network in DLBCL which impacts outcome, plays a tumour-suppressive role, and when lost presents potential for therapeutic intervention.

Author contributions statement

AB, HS, AE and TB conceived and carried out experiments and statistics. LB and RG collected clinical data. AM performed FISH analysis. EC and LD-B performed IHC and ISH assessments. MBC and CF performed NGS data. The other authors helped build the cohort. All authors were involved in writing the paper and had final approval of the submitted and published versions.
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