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Clinical and Pathological Features of Double-Hit and Triple-Hit High-Grade B-Cell Lymphomas: A Retrospective Study from Three Portuguese Tertiary Centers

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ABSTRACT

Background: High-grade B-cell lymphoma (HGBL) with rearrangements of *MYC* and *BCL2* and/or *BCL6,* called double and triple-hit lymphomas (DTH-HGBL), are lymphoid malignancies with inferior outcomes when treated with standard chemotherapy. The identification of DTH-HGBL cases is challenging, considering their variable clinical, morphologic, and immunohistochemical features.

Materials and Methods: Retrospective revision of medical data of patients diagnosed with DTH-HGBL confirmed by FISH, between January 2010 and January 2020, in three Tertiary Portuguese Hospitals (Coimbra Hospital and University Center, Portuguese Oncology Institute – Coimbra and Portuguese Oncology Institute – Porto). Pathological features, morphology, and immunohistochemical profile were evaluated by at least two experienced pathologists in hematopoietic and lymphoid neoplasms.

Results: The cohort included 24 patients: 33.3% triple-hit, 58.3%, *MYC/BCL2* double-hit and 8.3% *MYC/BCL6* double-hit*.* There was no gender predominance, with a median age of 62.5±14.3y, 33.3% were diagnosed as nodal disease, and 66.7% as extranodal. Morphologic features of DLBCL were present in 50% of cases, morphological features of both DLBCL and Burkitt lymphoma (DLBCL/BL) in 45.8% and 4.2% of blastoid morphology. Immunohistochemical evaluation, regarding the Hans algorithm, revealed a Germinal center (GC)/GC-like subtype in 83.3% of cases and a non-GC/non-*GC*-like subtype in 16.7%. MYC was positive in 42.9% and the median proliferative index was 80±12.4%.

Conclusion: DTH-HGBL has a very broad range of features. We consider that a cost-effective approach would be to perform cytogenetic analysis in DLBCL and DLBCL/BL cases with GC/GC-like subtype. MYC and BCL2 immunohistochemistry can be useful to identify patients who may benefit from more aggressive therapies, but not as tools for case selection for FISH.

Keywords: High-grade; B-cell; Lymphoma; Cytogenetics; Immunohistochemistry; Double-hit; Triple-hit

INTRODUCTION

In the revised 4th edition of WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, the classification of mature B-cell neoplasms includes the heterogeneous group of high-grade B-cell lymphoma (HGBL). It is a group of lymphoid malignancies with poor prognosis and inferior outcomes when treated with standard Rituximab,

Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone protocol (R-CHOP) chemotherapy or comparable therapies [1,2].

Genetic alterations, such as chromosomal translocations, are very common in various types of B-cell lymphomas. By definition HGBL are separated into cases associated with rearrangements of *MYC* and *BCL2* and/or *BCL6* rearrangements, so-called double and triple-hit lymphomas (DTH-HGBL), and into cases without these genetic alterations - HGBL-not otherwise specified (NOS) [1,3].

MYC gene is a proto-oncogene, encoding a nuclear protein with a vital role in cell proliferation, apoptosis, and cellular differentiation, located at chromosome 8q24[4]. Dysregulation of *MYC* is not exclusive to DTH-HGBL and has been described in multiple B-cell lymphomas: Burkitt lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), plasmablastic lymphoma (PBL), mantle cell lymphoma (ML), among others [5].

BCL2 gene is located at 18q21.33 and encodes an outer mitochondrial membrane protein that blocks apoptosis[6]. *BCL2* amplification and/or overexpression is present in various lymphoid neoplasms, seeming to be the key event to the development of follicular lymphoma (FL) and ML. It is also present in 30% of cases of DLBCL and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) [7].

BCL6 gene encodes a sequence-specific repressor of transcription, STAT-dependent, a 95 kDa protein, required in mature B cells during the germinal center phase, playing a key role in the suppression of *MYC* and *BCL2* expression in normal germinal center (GC) B cells. It is located at chromosome 3q27[8,9]. *BCL6* translocation is the most common genetic abnormality in DLBCL, present in 30–40% of cases.

There are reported cases of FL with rearrangement of *MYC* and *BCL2* and/or *BCL6* described in the literature, apparently with a worse prognosis than classical FL[10].

DTH-HGBL is not only diagnosed as a lymphoma *de novo* but also as a progression from low-grade B cell lymphomas, like FL[3].

Rearrangements of *MYC* and *BCL2* in lymphoid neoplasms were first described in 1988 in a case of acute pre-B-cell leukemia from a patient with a history of follicular lymphoma[11]. But, in light of the 4th edition of WHO classification, these cases should be classified as B-lymphoblastic leukemia/lymphoma with *MYC* and *BCL2* translocations, not as double-hit (DH) HGBL[1].

In their study from Herlev Hospital, in Denmark, Pedersen MØ *et al.* identified *MYC* and *BCL2* rearrangements in 11% of their cohort of 157 patients previously diagnosed with DLBCL and lymphomas with morphological features of both DLBCL and Burkitt lymphoma (DLBCL/BL)[12].

Around 80% of DH-HGBL harbor concurrent translocations in *MYC* and *BCL2*, and *MYC* and *BCL6* translocations were present in the remaining 20%[13].

Triple-hit (TH) HGBL incidence is still poorly known since they are uncommonly reported, and to our knowledge, the largest series published in 2018 by Huang *et al*. referred 40 patients. This series described clinicopathological features similar to double-hit HGBL[14,15].

Variable morphologic features are commonly described in DH-HGBL, they are more frequently reported as DLBCL-like, accounting for around 50% of cases; followed by DLBCL/BL morphology, accounting for a little less than 50%. Very uncommon cases have blastoid morphology, characterized by neoplastic cells resembling lymphoblasts[13,16].

Multiple studies have shown that morphological features and proliferation rates are not suitable for reliably identifying DTH-HGBL[17]. Most authors believe that MYC staining is not useful for screening, but other works suggest that immunohistochemical expression with MYC may be useful to select cases for cytogenetic analysis[1].

Cytogenetic studies are still expensive techniques, and not affordable to many Health Institutions. This unable many Pathology and Genetic Departments to perform these studies in all cases of B-cell lymphomas.

The great challenge of this classification is to identify, in everyday practice, the cases that are morphologically and immunohistochemically suspicious of harboring these genetic alterations since material costs associated with genetic analysis are a limiting factor in many countries.

In this work, we intend to study cases of DTH-HGBL with cytogenetic confirmation. These cases were all

diagnosed among Three Portuguese Tertiary Centers by experienced pathologists dedicated to hematopathology. The purpose of this study was to identify pathological features associated with rearrangements of MYC, BCL2, and BCL6 genes, helping to identify cases that may benefit from cytogenetics.

Due to the financial burden associated with large-scale Fluorescence in situ hybridization (FISH) analysis, pathologists face the issue of not being able to routinely request cytogenetic studies in cases suspicious of DTH-HGBL. In most countries, clinicians and pathologists must deal with the risk of missing some less characteristic cases of HGBL.

MATERIALS AND METHODS

This is a retrospective work, based on the revision of the electronic medical records.

The inclusion criteria encompass all patients diagnosed with DTH-HGBL with cytogenetic evidence of rearrangements of *MYC* with *BCL2* and/or *BCL6* rearrangements, over ten years (January 2010 to January 2020).

The exclusion criteria encompass all patients without a diagnosis of DTH-HGBL.

Our cohort is composed of cases diagnosed in three Tertiary Portuguese Hospitals, one university hospital (Coimbra Hospital and University Center), and two oncology centers (Portuguese Oncology Institute – Coimbra and Portuguese Oncology Institute – Porto) between January 2010 and January 2020.

Since this is retrospective work without clinical intervention, based solely on the revision of medical records with anonymized data, it was applied the Portuguese clinical research law (Law nº 21/2014 of 16 April amended by Law nº 73/2015 of 27 July). Exemption of Informed Consent in clinical studies without intervention (Article 6, nº 2) and Law nº 12/2005 of 26th of January (Article 19, no. 6).

Pathological features were evaluated by at least two experienced pathologists in hematopoietic and lymphoid neoplasms. All cases were classified under three morphologic categories described in the literature: DLBCL, DLBCL/BL, and blastoid.

Immunohistochemical (IHC) studies were performed using 3 µm tissue sections obtained from formalin-fixed and paraffin-embedded tissue, previously selected in hematoxylin-eosin (HE) slides by an experienced pathologist.

The panel of monoclonal antibodies included: Rabbit polyclonal antibody anti-human CD3 (1:150; Dako, Glostrup, Denmark), mouse monoclonal antibody anti-human CD20, clone L26 (1:150; Dako, Glostrup, Denmark), mouse monoclonal antibody anti-human CD5, SP19 (ready-to-use; Ventana Medical Systems, Tucson, United States), mouse monoclonal antibody anti-human CD10, 56C6 (1:200; Dako, Glostrup, Denmark), mouse monoclonal antibody anti-human BCL-2, SP66 (ready-to-use; Ventana Medical Systems, Tucson, United States), mouse monoclonal antibody anti-human BCL-6, GI191E/A8 (ready-to-use; Cell Marque Corporation, Rocklin, United States), mouse monoclonal antibody anti-human MUM1, MRQ-43 (ready-to-use; Cell Marque Corporation, Rocklin, United States), mouse monoclonal antibody anti-human C-MYC, clone 9E10 (Diagnostic BioSystems; Pleasanton, United States) and mouse monoclonal antibody anti-human Ki-67, MIB-1 (1:200; Dako, Glostrup, Denmark).

CD10, BCL6, and MUM1 were labeled as positive if at least 30% of neoplastic cells were stained, according to the Hans algorithm for DLBCL. (18) MYC was considered positive when >40% of the tumor cells had nuclear staining, and BCL2 when there were≥50% stained neoplastic cells[19,20].

FISH was performed using 3 µm tissue sections, obtained from formalin-fixed and paraffin-embedded blocks, in areas previously selected in HE by an experienced pathologist.

Interphase FISH analysis was performed using split signal sections with dual-color break-apart probes for *MYC* (8q24.21, ZytoVision, Bremerhaven, Germany), *BCL6* (3q27.3, ZytoVision, Bremerhaven, Germany), and *BCL2* (18q21.33, ZytoVision, Bremerhaven, Germany) according to the manufacturer. At least 100 nuclei signals were analyzed, being considered *MYC, BCL2*, and *BCL6* rearrangements when abnormal signals were present in >10% of all assessed nuclei.

Metric variables were described and compared using Student's t-tests. Categorical variables were

described by absolute and relative frequencies, and the distributions were compared using Chi-square tests. For IHC studies (MYC, BCL2, and BL6), the sensitivity was determined according to the percentage of positive IHC that was confirmed by FISH in the total population. The specificity was assessed with the percentage of negative IHQ that was also negative by FISH in the total population. The positive predictive value (PPV) was defined by the probability of a positive result on IHC and a positive result on FISH. The negative predictive value (NPV) was defined as the probability of a negative result on IHQ and a negative result on FISH.

A two-sided p-value <0.05 was considered representative of statistical significance. Statistical calculations were performed with SPSS (Version 22.0, Chicago, IL).

RESULTS

The cohort (table 1) included 24 patients diagnosed with DTH-HGBL: 12 (50.0%) males and 12 (50.0%) females, with a median age of 62.5±14.3 years (range 38-87).

In eight (33.3%) cases, diagnosis resulted from a lymph node specimen, being classified as a nodal disease and in 16 (66.7%) cases an extranodal site was sampled, being considered as extranodal disease.

Among the 16 cases diagnosed as extranodal DTH-HGBL, 7 (43,8%) were sampled in the skin/deep soft tissue, 5 (31,3%) in the retroperitoneum/abdominal cavity, 1 (6,3%) in the small bowel, and 1 (6,3%) in the pancreas.

Cytogenetic studies (*Figure 1*) revealed 8 (33.3%) TH-HGBL and 16 (66.7%) DH-HGBL: 14 (58.3%) with *MYC/BCL2* rearrangements and 2 (8.3%) with translocations involving *MYC/BCL6*.

Regarding morphologic features (*Figures 2 and 3)*, 12 (50.0%) cases were diagnosed as DLBCL, 11 (45.8%) as DLBCL/BL, and one (4.2%) case was classified as blastoid.

Considering IHC evaluation, due to material scarcity, MYC was inconclusive in three of the 24 cases, three cases of DH-HGBL, two with *MYC/BCL2* alterations, and one case with rearrangements of *MYC/BCL6*. These cases were excluded from the statistical analysis of MYC immunohistochemistry, so the values reported are referring to a cohort of 21 cases. MUM1 immunohistochemistry was also inconclusive in one case of DH-HGBL with *MYC/BCL2* anomalies, also owing to material scarcity, leaving us with a cohort of 23 cases in the statistical evaluation of MUM1.

The Immunohistochemical profile of the whole cohort of DTH-HGBL revealed positivity for BCL2 in 20 (83.3%) cases, BCL6 in 23 (95.8%) cases, CD10 in 21 (87.5%) cases, and MUM1 in 12 (52.2%), and MYC was positive in 9 (42.9%) cases.

The median proliferative index, evaluated by Ki67, was 80±12.4% (range 50-100).

Resorting to Hans algorithm, all cases were classified as GC/GC-like subtype and non-GC/non-GC-like subtype: 20 (83.3%) cases were GC/GC-like subtype and four (16.7%) were non-GC/non-GC-like. Although the classification of GC and non-GC is only well established for DLBCL, we considered a GC-like and non-GC-like classification in DLBCL/BL and blastoid cases.

Considering the cases of DLBCL, 9 (81.8%) were classified as GC and 2 (18.2%) as non-GC. Applying this concept to DLBCL/BL cases, 10 (83.3%) were GC-like and 2 (16.7%) were non-GC-like. The one (100%) case of blastoid morphology would be classified as GC-like (*Table 1*).

IHC evaluation showed a sensitivity of 86.4% and a specificity of 50% for BCL2 and a sensitivity of 100% and a specificity of 7% for BCL6. Regarding MYC, despite all cases being positive in FISH, only 9 (42.9%) cases of the 21 tested on IHC were considered positive.

The PPV for BCL2 was 95% and for BCL6 was 43.5%.

Regarding our sub-cohort of TH-HGBLs (eight cases), the median age was of 58±12.3 years (38-78), is constituted of five (62.5%) males and three (37.5%) females. Disease location at the time of diagnosis was nodal and extranodal in three (37.5%) and five (62.5%) cases, respectively. Morphologic evaluation disclosed three (37.5%) cases of DLBCL and five (62.5%) of DLBCL/BL. Immunohistochemistry revealed positivity for BCL2 in seven (87.5%) cases, BCL6 in eight (100%), CD10 in seven (87.5%), MUM1 in five (62.5%), MYC in four (50%) and median Ki67 was 80±15.5% (60-100).

Concerning the sub-cohort of double-hit *MYC/BCL2* HGBLs (14 cases), the median age was 68±15.4y 39-

87, is composed of six (42.9%) males and 8 (57.1%) females. The sample site at the time of diagnosis was nodal for four (28.6%) and extranodal for 10 (71.4%) cases. Morphologic evaluation revealed nine (64.3%) DLBCL and 5 (37.5%) DLBCL/BL. Immunohistochemistry was positive for BCL2 in 12 (85.7%) cases, BCL6 in 13 (92.9%), CD10 in 12 (85.7%), MUM1 in six (46.1%), MYC in four (33.3%) and median Ki67 was 80±11.5% (50-95).

Considering the two cases of double-hit *MYC/BCL6* HGBL, the median age was 58y (47y and 69y), represented by one (50%) male and one (50%) female. One (50%) was diagnosed with material sampled from lymph nodes and one (50%) from an extranodal site. One (50%) was of DLBCL morphology and one (50%) displayed a blastoid appearance. Immunohistochemistry: BCL2 positivity in one (50%), BCL6 in 2 (100.0%), CD10 in one (50%), MUM1 in one (50%) and MYC was positive in one case (the other one was not available due to material scarcity). Both cases had a Ki67 of 90%.

There was no association between location and morphology (p=0.121) and between location and cytogenetic classification (p=0.786). This was also observed with gender and morphology (p=0.1) and with gender and cytogenetic analysis (p=0.675).

The classification DTH-HGBL as GC/GC-like and non-GC/non-GC-like was not associated with location (p=0.699), gender (p=0-295), morphology (p=0.897) and cytogenetic classification (p=0.415).

Results are summarized in *Table 2* for easier consultation.

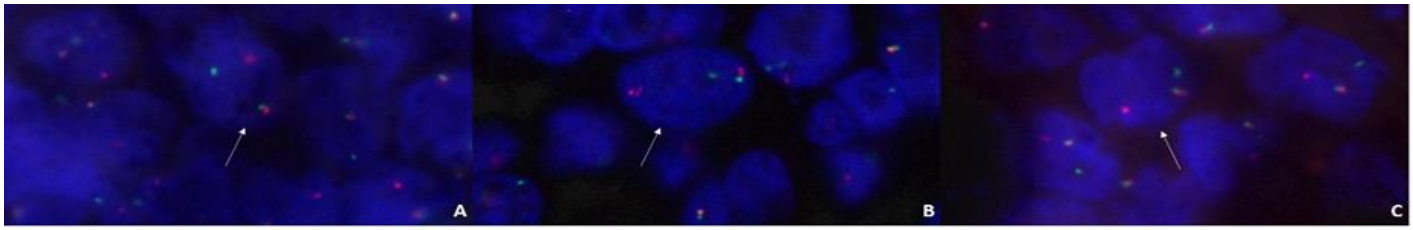

Figure 1: FISH signal patterns in paraffin tissue section using dual-color break-apart probes. A - *MYC* dual-probe; B - *BCL2* dual-probe; C - *BCL6* dual probe. The translocation is recognized by the presence of single green and red signals. Normal allele was represented by a set of colocalizing yellow signals and the presence of a monoallelic split with one single green and one red signal

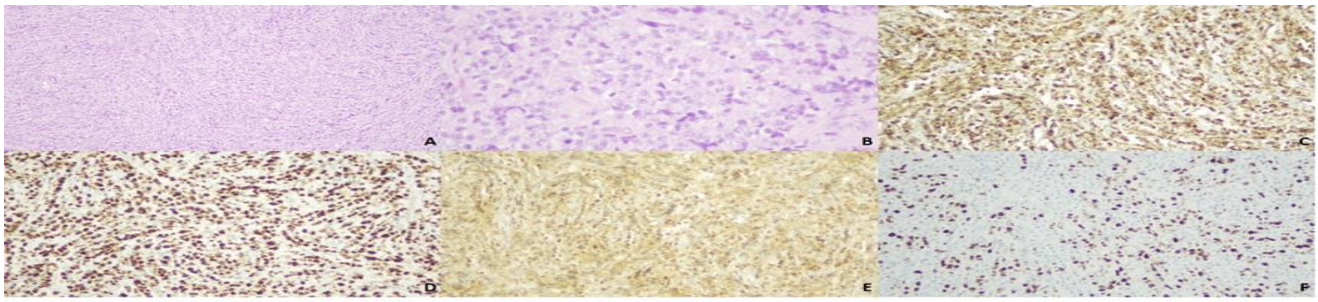

Figure 2: A and B – High grade B cell lymphoma (HGBL) with morphologic features of diffuse large B-cell lymphoma (H&E 100x / 400x); C – Diffuse immunohistochemical (IHC) expression for BCL2 (200x); D – Diffuse IHC expression for BCL6 (200x); E – Diffuse IHC expression for MYC (200x); F - IHC expression for Ki67 in around 50% of neoplastic cells (200x)

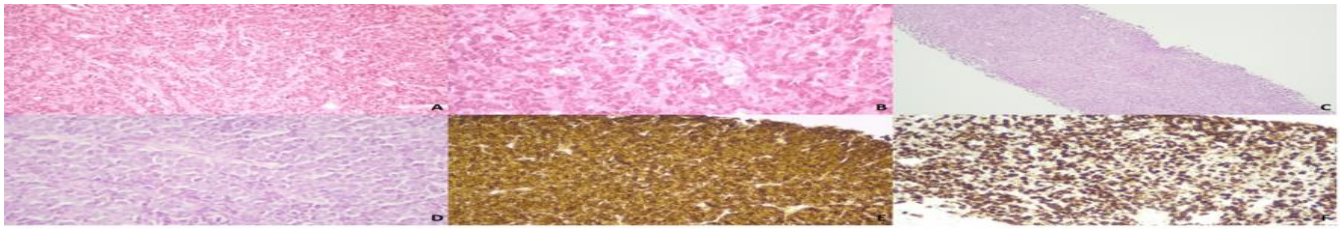

Figure 3: A and B – High grade B cell lymphoma (HGBL) with blastoid morphologic features (HE 200x / 400x); C and D – HGBL with intermediate features of Burkitt lymphoma and diffuse large B-cell lymphoma (HE 100x / 400x); F – Diffuse immunohistochemical (IHC) expression for BCL2 (200x); E – Diffuse IHC expression for BCL2 (200x); F - IHC expression for Ki67 in around 80% of neoplastic cells (200x)

Table1. Relation between morphologic features and immunohistochemical classification on subtype germinal center (GC) or GC-like, and non-GC or non-GC-like

	DLBCL/BL (n=11)	**DLBCL (n=12)**	**Blastoid (n=1)**
Subtype			
GC/GC-like	9 (81.8%)	10 (83.3%)	1 (100%)
Non-GC/non-GC-like	2 (18.2%)	2 (16.7%)	0 (0%)

Table 2. Summary of results

	Triple-hit (n=8)	**Double-hit *MYC/BCL2* (n=14)**	**Double-hit *MYC/BCL6* (n=2)**	**HGBL (n=24)**
Median age	58±12.3y	68±15.4y (39-87)	58y (47-69)	62.5±14.3y
(range)	(38-78)			(38-87)
Gender (M: F)	5:3	6:8	1:1	12:12
Anatomical location (n)				
Nodal	3 (37.5%)	4 (28.6%)	1 (50%)	8 (33.3%)
Extranodal	5 (62.5%)	10 (71.4%)	1 (50%)	16 (66.7%)
Morphology				
DLBCL/BL	5 (62.5%)	5 (35.7%)	1 (50%)	11 (45.8%)
DLBCL	3 (37.5%)	9 (64.3%)	0 (0%)	12 (50.0%)
Blastoid	0 (0%)	0 (0%)	1 (50%)	1 (4.2%)
Immunohistochemistry (n of positive cases)				
BCL2	7 (87.5%)	12 (85.7%)	1 (50%)	20 (83.3%)
BCL6	8 (100%)	13 (92.9%)	2 (100%)	23 (95.8%)
CD10	7 (87.5%)	12 (85.7%)	1 (50%)	21 (87.5%)
MUM1	5 (62.5%)	6 (46.1%)*	1 (50%)	12 (52.2%)*
MYC	4 (50.0%)	4 (33.3%)*	1 (100%)*	9 (42.9%)*
Median Ki67	80±15.5%	80±11.5%	90%	80±12.4% (50-100)
(range)	(60-100)	(50-95)	(90)	
Subtype				
GC	7 (87.5%)	12 (85.7%)	1 (50%)	20 (83.3%)
non-GC	2 (12.5%)	2 (14.3%)	1 (50%)	4 (16.7%)

* Due to material scarcity, immunohistochemistry with MYC was inconclusive in three cases. MUM1 was inconclusive in one case, also owing to material scarcity.

DISCUSSION

DTH-HGBL is a heterogeneous group, requiring a correct diagnosis since they are associated with worse outcomes and potential benefits from more aggressive treatment.

In this cohort, the median age at the time of diagnosis was 62.5y, similar to those described in the literature regarding DLBCL and HGBL, since both occur mostly in the 7th decade of life[24]. In the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues book, a slight predominance in male patients is reported, although not observed in this cohort, since there was no gender predilection[1].

Regarding morphology, there were no significant differences between DH (*MYC/BCL2* and *MYC/BCL6*) and TH-HGBLs. This cohort has overlapping features with other reported series: half of the cases of HGBL have morphologic features of DLBCL, slightly few have features of DLCBL/BL and only one case has the very uncommon blastoid morphology[13,16].

The IHC profile (*Table 2)* revealed that the great majority of DTH-HGBL are of GC/GC-like subtype, supporting the suggestion of performing cytogenetic analysis in these cases.

If we considered only the cases with DLBCL morphology, 83.3% were GC subtype, consisting of the vast majority of cases. In the remaining cases, although there is no definition for GC or non-GC classification in DLBCL/BL morphology, if we consider a GC-like subtype, we can observe a clear predominance of this subtype (81.8%). This data is similar to the one already published, referring that DH-HGBL is of GC subtype in 90% of cases[22].

The proliferative index is globally high, with a median of 80%, although with a relatively broad range and the lowest value of 50%. Some papers report that the proliferative index is not a useful tool to identify DTH-HGBL, since some cases may have very low values (<30%)[1,25]. Nevertheless, in this series proliferative index was considerably high in all cases, so it could be an additional indicator for FISH analysis, particularly in cases with GC/GC-like phenotype.

MYC immunohistochemistry was positive in a little over 40% of cases of DTH-HGBL, decreasing to one-third if we consider only the cases of double-hit *MYC/BCL2.* Although it does not seem to be a good tool to select cases for FISH, IHC evaluation of MYC may be useful as a prognostic tool since those cases with co-expression of BCL2 appear to have worse outcomes when treated with standard therapy [26].

In their study, Huang et al. analyzed the IHC features of a series of 210 cases of DTH-HGBL with results very close to the ones observed in this cohort. They reported CD10 positivity in 100% of triple-hit, 98% of *MYC/BCL2* double-hit, and 75% of *MYC/BCL6* double-hit. BCL6 was expressed in 82% of triple hits, 92% of *MYC/BCL2,* and 100% of *MYC/BCL6.* Positivity for BCL2 was seen in 95% of triple hit, 91% of *MYC/BCL2,* and 80% of *MYC/BCL6.* MYC was positive in 74% of triple hits, 80% of *MYC/BCL2,* and 67% of *MYC/BCL6.* The median proliferative index, evaluated by Ki67, was 80% in triple hit, 80% in *MYC/BCL2,* and 80% in *MYC/BCL*[27].

Considering the correlation between immunohistochemistry and cytogenic analysis, we observed a PPV for BCL2 of 95% and BCL6 of 43.5%.

Some authors suggest performing FISH testing on all DLBCL with GC phenotype, which accounts for almost 60% of DLBCL[21]. While others propose cytogenetic analysis in all cases of DLBCL[22].

This last approach is very controversial since DLBCL constitutes a great number of non-Hodgkin lymphomas, accounting for 30-40% of all B-cell lymphomas in western countries, thus encompassing high financial costs[23]. This is particularly evident if we consider the low frequency of these chromosomal anomalies since, for instance, DH-HGBL has been reported as accounting for around 4% of DLBCL cases[22].

It is imperative to identify DTH-HGBL features, in a way that may lead to cost-effective diagnostic procedures, without losing the commitment of sensitivity and specificity.

Although we have included cases from three tertiary centers and we have obtained similar data to the published literature, this is still a small cohort. An investigation with a greater number of cases is required.

CONCLUSION

In the light of current data and of others published in the literature, we think that it will be more cost-effective to perform cytogenetic analysis in cases of

GC subtype DLBCL and DLBCL/BL with GC-like subtype, particularly if a high proliferative index is present.

MYC and BCL2 immunohistochemistry may be useful in prognosis and FISH case selection to identify patients who may benefit from more aggressive therapies.

Although this work has provided some valuable data to very uncommon entities, more studies are needed to draw more robust conclusions and recommendations.

A prospective study with cytogenetic analysis in a broader range of B-cell lymphoma cases could be beneficial, maybe allowing the selection of patients to include in clinical trials and discover more effective treatments.

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