Oral and oropharyngeal diffuse large B-cell lymphoma and high-grade B-cell lymphoma: A clinicopathologic and prognostic study of 69 cases

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Objective. The objective of this study was to describe the clinicopathological, molecular, and prognostic features of oral/oropharyngeal diffuse large B-cell lymphoma (DLBCL) and high-grade B-cell lymphoma.

Study Design. All cases were retrieved from 7 Brazilian institutions. Immunohistochemical reactions were performed to confirm the diagnoses and to categorize the tumors. In situ hybridization was used to detect Epstein-Barr virus (EBV) and fluorescence in situ hybridization was used to identify gene rearrangements.

Results. Most cases involved the oral cavity (76.8%). Males and females, with a mean age of 60 years, were evenly affected. Tumors mostly presented as painful swellings. Forty cases represented germinal center B-cell type (58%). Five cases presented double-hit translocation and 3 harbored rearrangement for *MYC/BCL2/BCL6*. EBV was detected in 3 cases (4.3%). The 5-year overall survival was 44.4%. Female sex, presence of pain and ulcer, microscopic "starry sky pattern" and necrosis, co-expression of c-Myc/Bcl2, and translocation of *MYC* were associated with a lower survival in univariate analysis ($P = .05$, $P = .01$, $P = .01$, $P = .03$, $P = .05$, $P = .006$, $P = .05$, respectively).

Conclusion. Patients affected by oral/oropharyngeal DLBCL have a low survival rate. High-grade B-cell lymphoma (17.7%) and EBV−positive DLBCL, not otherwise specified (4.3%) account for a small number of cases. (Oral Surg Oral Med Oral Pathol Oral Radiol 2021;131:452−462)

Diffuse large B-cell lymphoma (DLBCL), not otherwise specified (NOS) is a neoplasm of large B cells arranged in a diffuse growth pattern.[1] It is characterized as a heterogeneous malignancy because its clinical presentation and outcome are remarkably variable, reflecting its biological and pathogenetic diversity.[2,3] DLBCL, NOS has an annual incidence of over 100,000 cases worldwide, representing 25% to 35% of adult non-Hodgkin lymphomas in developed countries, and 42.5% in developing nations.[2,4,5] Different etiologies have been

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postulated for this tumor but with no consensus. However, some cases develop in an underlying immunodeficiency basis, and the Epstein-Barr virus (EBV) infection has been identified in a varying number of cases, which are now termed EBV−positive DLBCL, NOS.[5]

Clinically, DLBCL, NOS usually manifests as a rapidly growing tumor, which affects extranodal sites in over 40% of cases.[1,6] In the head and neck region, the most common site of DLBCL, NOS is the Waldeyer's ring, followed by the paranasal sinuses and the oral cavity, where the gingiva and palate appear to be the most affected locations.[7,8]

Recently, we extensively reviewed the literature to describe the clinicopathologic features of oral DLBCL, NOS. Nevertheless, most of the currently available data are based on individual case reports or small clinical series that include cases affecting different areas of the head and neck, with limited data regarding the immunohistochemical diagnosis and the survival outcomes.[8]

Moreover, the last revised version of the World Health Organization's (WHO) *Classification of Lymphoid Neoplasms* defined a new subset of DLBCL, which are now termed as high-grade B-cell lymphomas (HGBLs). This group encompasses aggressive and mature B-cell neoplasms, which may be subclassified according to the presence of chromosomal rearrangements involving *MYC*, *BCL2*, and/or *BCL6* genes, giving rise to the so-called double-hit and triple-hit lymphomas. In addition, the cases that are defined as HGBL, NOS tend to exhibit intermediary microscopic features between DLBCL and Burkitt lymphoma, or even appear blastoid, but do not harbor genetic alterations in *MYC*, *BCL2*, and/or *BCL6* genes.[9,10] The incidence and clinical implications of this novel category are only now becoming more evident. Despite recent case reports of HGBL affecting the oral cavity[11] and oropharynx,[12] the clinicopathological features of the tumors involving these regions remain largely unknown.

Therefore, the purpose of this study is to comprehensively describe the clinicopathological characteristics, immunohistochemical findings, EBV status, and molecular features of a large sample of oral and oropharyngeal DLBCL, NOS and to investigate their survival rate. In addition, we aim to determine the frequency of HGBL present in this sample and describe its clinical and pathological aspects.

MATERIAL AND METHODS

This study was approved by the Ethical Committee of the Piracicaba Dental School, University of Campinas, Piracicaba, Brazil (Process No. 67128417.4.0000.5418).

Study population

All cases diagnosed as oral and oropharyngeal DLBCL, NOS between January 2001 and December 2019 were retrospectively retrieved from the pathology files of 7 Brazilian institutions: Piracicaba Dental School of the University of Campinas (Piracicaba), School of Dentistry of the Universidade Federal de Minas Gerais (Belo Horizonte), Oral Pathology Service of the João de Barros Barreto University Hospital (Belém), Federal University of Rio Grande do Sul (Porto Alegre), School of Dentistry of the Federal University of Rio de Janeiro (Rio de Janeiro), Dental School of the State University of Rio de Janeiro (Rio de Janeiro), and Private Pathology Service (Natal). Formalin-fixed, paraffin-embedded tissues (FFPE) were obtained and new histologic sections were stained with hematoxylin-eosin for microscopic description and diagnostic confirmation by at least 2 oral pathologists, following the current WHO *Classification of Lymphoid Neoplasms*.[5] The clinicopathologic features were retrieved from the patients' medical files and included age, sex, tumor location, clinical presentation, time of evolution, imaging features, treatment, status at last follow-up (dead or alive), and follow-up period. The overall survival rate was defined as the time from the date of diagnosis to the date of the patient's last follow-up or death.

Immunohistochemistry

Immunohistochemical reactions were performed in 3-μm sections of FFPE tissues that were dewaxed with xylene and then hydrated in a descending ethanol series. The endogenous peroxidase activity was blocked with 10% hydrogen peroxide in a single bath for 15 min. After washing in phosphate-buffered saline (pH 7.4), the sections were incubated for 2 h with primary antibodies and then exposed to high-sensitivity horseradish peroxidase reagents (ADVANCE, Dako, Capinteria, CA) and diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St Louis, MO). The slides were counterstained with Carazzi hematoxylin (Piracicaba Dental School, University of Campinas) for 3 min. Histologic sections with positive control were used for each antibody, and the negative control was acquired by omitting the specific primary antibody.

All cases were submitted to the same immunohistochemical reactions for the diagnosis of DLBCL, NOS, which initially included LCA, CD3, CD20, and cyclin D1, followed by CD10, Bcl6, MUM1, Bcl2, and Ki67.

Statement of Clinical Relevance

Oral and oropharyngeal diffuse large B-cell lymphomas are aggressive neoplasms with a low 5-year survival rate (44.4%). The recently described high grade B-cell lymphoma (double/triple-hit) accounted for 17.7% of the previously diagnosed diffuse large B-cell lymphomas not otherwise specified.

The expression of c-Myc protein was investigated in all cases with available tissue sections (55 cases). Other antibodies were also used when necessary to elucidate the diagnosis of some cases, such as pan-cytokeratin (AE1/AE3), vimentin, plasma cell, CD138, CD79a, PAX5, TdT, CD45 RO, CD56, CD68, CD30, CD43, and CD5. Detailed information about immunohistochemical antibodies and the methods applied in the reactions are available in Supplemental Table S1-S5 (available at [URL/link]).

Immunohistochemistry quantification

All immunohistochemical reactions were descriptively evaluated according to the cellular compartment and cellular population expressing the proteins. Cutoff values of 50% of neoplastic cells expressing Bcl2 in the cytoplasm and 40% of c-Myc nuclear staining were used to determine positivity for these markers, according to the current WHO *Classification of Lymphoid Neoplasms*.[5] The proliferative index was obtained by calculating the percentage of malignant cells with nuclear staining for Ki67 among 1000 tumor cells using a high-power view in 5 hot-spot fields. The mean value was used to classify them as low or high proliferative rate. Subclassification of the cases according to the Hans et al.[13] algorithm for DLBCL, NOS was performed combining the cytoplasm staining of CD10 and the nuclear staining of Bcl6 and MUM1, using a cutoff value of 30% for each antibody. The algorithm proposed by Muris et al.[14] was also analyzed, considering the combined expression of Bcl2, CD10, and MUM1.

In situ hybridization for EBV detection

All cases were submitted to in situ hybridization (ISH) to detect EBV, in 3-μm sections of FFPE tissues. A fluorescein-labeled peptide nucleic acid probe complementary to 2 nuclear-encoded RNAs (EBER; Y5200, Dako, Glostrup, Denmark) was hybridized at 55˚C for 90 min and then labeling was performed using the peptide nucleic acid ISH detection kit (K5201, Dako). One case of extranodal NK/T-cell lymphoma, nasal type, was used as a positive control. Carazzi hematoxylin was used for subsequent counterstaining. Cases considered positive for EBV presented a dark blue staining in the nuclei of the tumor cells.

Fluorescence in situ hybridization for MYC, BCL2, and BCL6 rearrangements

Fluorescence ISH analysis was performed to detect *MYC*, *BCL2*, and *BLC6* gene translocations. Histologic sections of 4-μm FFPE tissues were dewaxed in xylene and rehydrated in ethanol series. The denaturation procedure was carried out on a hot plate at 95˚C for 10 min and the samples were incubated in a humid chamber shielded from light at 37˚C for approximately 16 h. Dual-color break-apart probes (MYC/8 q24 and BCL6/3 q27) and dual-color fusion translocation probes (BCL2/18 q21) were used (Abbott Laboratories, Des Plaines, IL). The cells were hybridized with *MYC*, *BCL2*, and *BCL6* probes and nuclei were counterstained with 4′,6-diamidino-2-phenylindole.

Analysis was performed using an Olympus BX 41 fluorescence microscope combined with the Case Data Manager 5.0 (ASI, Carlsbad, California, USA) Olympus BX 41 fluorescence microscope (Olympus Corporation, Tokyo, Japan) program, where 100 cells/case were randomly counted regarding the probe signals in each allele of the gene that was investigated.

Statistical analysis

Chi-square and Fisher's exact tests were used to investigate associations between clinicopathologic features and patients' status (alive or dead), as well as the immunohistochemical markers and clinicopathologic parameters. The Kaplan-Meier method was used to calculate survival curves, and the differences between the curves were investigated using the log-rank univariate test to identify potential prognostic factors. All variables that achieved significance in the univariate analysis and treatment modality were included in a multivariate model created using the Cox proportional hazard test to identify independent prognostic factors. SPSS (IBM®, New York, USA) Version 22.0 was used, and $P \leq .05$ was considered statistically significant.

RESULTS

Demographic characteristics

One hundred twenty-two cases diagnosed as DLBCL, NOS were initially retrieved from the assessed services' files; however, 44 cases were excluded due to lack of clinical information, lack of available sections to confirm the diagnosis, or absence of paraffin blocks or histologic sections. Nine tumors showed positivity for cyclin D1 and were reclassified as mantle cell lymphomas. Therefore, 69 cases remained in the present study for analysis.

The clinicopathological features of all 69 cases included in this study are summarized in Table I. The cases were uniformly distributed among sexes, comprising 35 males (50.7%) and 34 females (49.3%). Most patients were diagnosed in the seventh decade of life (mean age = 60 years; range, 23-88 years). The majority of our sample consisted of oral cavity lymphomas (53 cases; 76.8%), whereas oropharyngeal cases accounted for 16 cases (23.2%). The hard palate and the jaw bones were the most frequently affected sites (23 and 11 cases, respectively), followed by the gingiva (6 cases), buccal mucosa (5 cases), retromolar trigone (3 cases), alveolar ridge mucosa (2 cases), the floor of the mouth (2 cases), and lower lip (1 case). Moreover, 4 cases presented an extension to the maxillary sinus. The cases of oropharynx

Table I. Demographic and clinicopathological features of the 69 cases investigated in this study

Clinicopathological variables	*N = 69*	*Total (%)*
Sex		
Female	34	49.3
Male	35	50.7
Age (mean age: 60 years)		
<60 years	26	37.7
>60 years	43	62.3
Site		
Oral cavity	53	76.8
Oropharynx	16	23.2
Symptoms		
Pain	18	26.1
Asymptomatic	22	31.9
ND	29	42.0
Swelling		
Presence	49	71.0
Absence	14	20.3
ND	6	8.7
Ulcer		
Presence	29	42.0
Absence	29	42.0
ND	11	15.9
Bleeding		
Presence	3	4.3
Absence	17	24.6
ND	49	71.0
Bone destruction		
Presence	9	13
Absence	15	21.7
ND	45	65.2
Treatment		
CHOP	13	18.8
R-CHOP	5	7.2
Others*	15	21.7
ND	36	52.2
Status		
Alive	19	27.5
Dead	22	31.9
ND	28	40.6

ND, information not described; *CHOP*, cyclophosphamide, doxorubicin, vincristine, and prednisone; *R-CHOP*, rituximab + CHOP.
*Others indicates cases for which the chemotherapy scheme was not specified.

included the soft palate (4 cases), palatine tonsil (3 cases), and base of the tongue (4 cases). Five cases did not report the specific oropharyngeal site.

Clinical features

The most common clinical presentation was an asymptomatic swelling (49 cases; 71%), although pain was reported in 18 cases (26.1%). Additional clinical signs included bleeding (3 cases; 4.3%) and bone destruction (9 cases; 13.0%; Table I). When radiograph and/or computed tomography images were available for intraosseous lymphomas, these cases demonstrated ill-defined radiolucent/hypodense images, causing cortical bone expansion and destruction. Four cases were shown to involve the maxillary sinus, and 1 case showed extension to the orbital cavity. Patients more frequently reported rapidly growing tumors, with less than 1-month duration, although some cases were reported with almost 1 year of evolution (4 cases; 5.8%).

Microscopic findings

The affected tissues exhibited effacement of their architecture by the diffuse infiltration of neoplastic cells, which permeated the surrounding normal structures such as muscles, vessels, bone, and adipose tissue, exhibiting extensive areas of necrosis in most of the cases (63 cases; 91.3%; Figure 1). Ulceration of the overlying mucosa was also a common finding (30 cases; 43.5%). Eighteen cases (26.1%) presented the so-called "starry sky" appearance, demonstrating a variable number of tingible body macrophages with phagocytosed cell debris.

Centroblasts were the predominant cell type in 50 cases (72.5%) and consisted of large noncleaved cells with round to oval vesicular nuclei, and multiple small nucleoli. The immunoblasts predominated in 19 cases (27.5%) and exhibited round to oval vesicular nuclei with a single, prominent, and centrally located nucleolus. Nevertheless, the admixture of these 2 cell types was observed in all tumors, together with smaller and more hyperchromatic centrocyte-like cells. Moreover, cellular atypia, such as irregular nuclear foldings, coarse chromatin, and giant or bizarre nuclei were also present. Atypical mitotic figures were commonly observed, and the mean mitotic ratio in our sample was 4.4 mitoses per high-power field (HPF), ranging from 1 to 15.2 mitoses/HPF (Table II).

Immunohistochemical and EBV ISH results

All cases showed positivity for LCA and CD20 antibodies. Cyclin D1 was negative in all 69 cases. CD3 was also negative in the tumor cells, but exhibited positivity in a variable number of small reactive lymphocytes. Bcl2 cytoplasmic staining was positive in 47 cases (68.1%), and c-Myc was expressed in 22 cases (40.0%). Two tumors co-expressed Bcl2 and c-Myc (3.6%), and were classified as double expressers. Twenty-five cases were positive for CD10 protein (36.2%); MUM1 was expressed in 44 cases (63.8%), and Bcl6 was positive in 51 cases (73.9%). According to the Hans et al.[13] algorithm for DLBCL, NOS, 40 cases were classified in the germinal center B-cell (GCB) type group (58.0%), and 29 cases were categorized into the activated B-cell (non-GCB) type group (42.0%). According to the Muris et al.[14] algorithm, 49 cases were placed in the favorable prognosis group (71.0%), and 20 cases (29.0%) had an unfavorable prognosis. The mean proliferative index obtained with Ki67 was 56.2%, ranging from 25% to 90% (Table II).

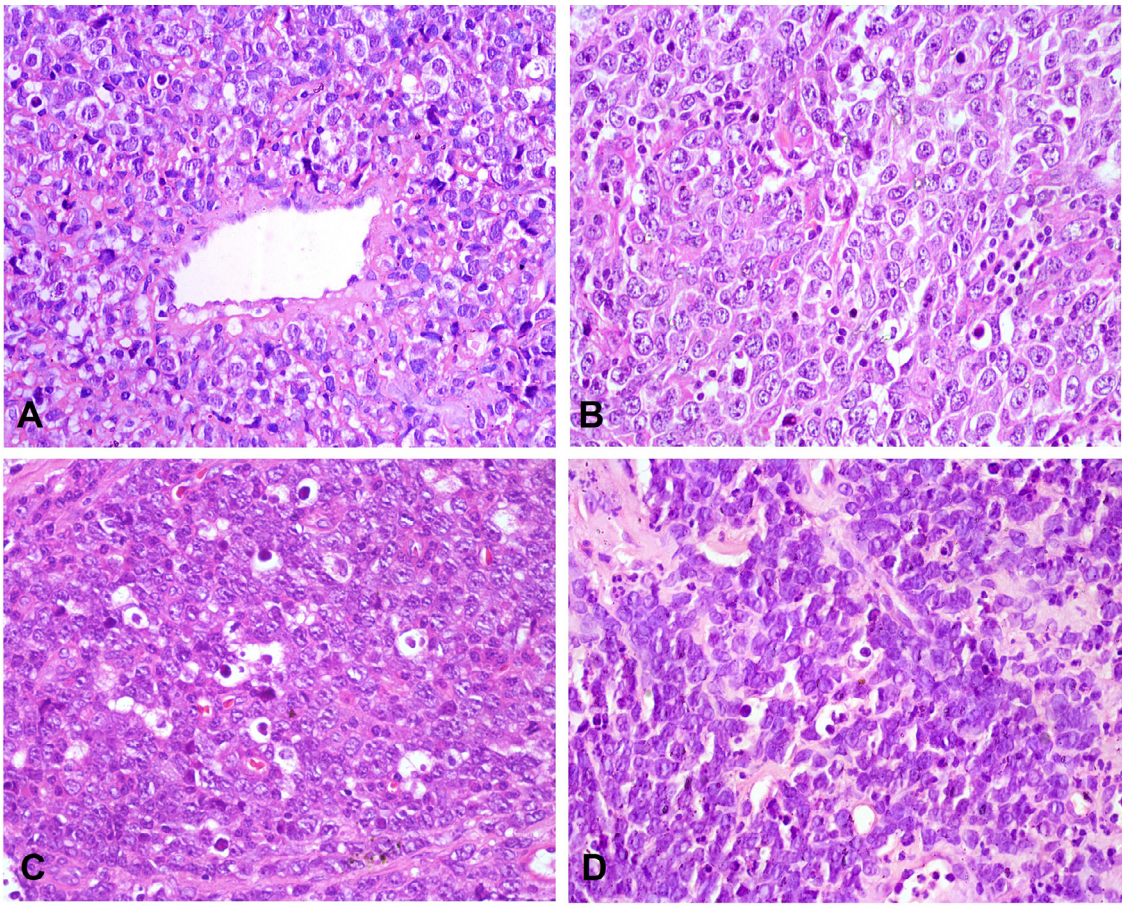

Fig. 1. Microscopic features of diffuse large B-cell lymphoma, not otherwise specified, and high-grade B-cell lymphoma. **(A)** Diffuse large B-cell lymphoma, not otherwise specified was characterized by a diffuse lymphoid infiltrate of large neoplastic cells. In this image an angiocentric growth pattern can be observed [hematoxylin-eosin (H&E], 200×]. **(B)** Neoplastic cells comprised centroblasts containing large noncleaved cells with round and vesicular nuclei with multiple small nucleoli and immunoblasts exhibiting oval and round vesicular nuclei with a prominent nucleolus. Apoptotic bodies are also noted (H&E, 400 ×). **(C)** Triple-hit high-grade B-cell lymphoma exhibited a diffuse growth pattern with the so-called starry sky appearance (H&E, 100 ×). **(D)** The neoplasm showed a predominance of medium to large centroblasts with vesicular nuclei and tingible body macrophages with phagocytosed cell debris. Atypical mitotic figures are also observed (H&E, 400 ×).

EBV was detected in only 3 cases, all located in the oral cavity (4.3%); these were subcategorized as EBV−positive DLBCL, NOS. These cases included 2 females and 1 male patient, aged 54, 23, and 29 years old, respectively. Only 1 case manifested as a painful swelling, and 2 cases were asymptomatic. No bone involvement was reported. Centroblasts were the predominant cell type in all 3 tumors, and the mean mitotic ratio was 7.0 mitoses/HPF (range, 2.2-10.2). The mean proliferative index was 57.8%, ranging from 44.6% to 69.3%. One case has been previously reported.[15]

Genetic rearrangements of *MYC*, *BCL2*, and *BCL6* genes

Reactions were performed in 45 cases to investigate the presence of chromosomal abnormalities. As shown in Table II, single rearrangements of *BCL2*, *BCL6*, and *MYC* genes were detected in 11 (24.4%), 5 (11.1%), and 11 tumors (24.4%), respectively. Double translocations of *MYC/BCL2* and *MYC/BCL6* were observed in 3 (6.7%) and 2 (4.4%) cases, respectively. None of the cases presented simultaneous translocations of both *BCL2* and *BCL6* genes. Three cases (6.7%) presented simultaneous rearrangements of *MYC/BCL2/BCL6* genes. The cases with double and/or triple translocations were then reclassified as HGBL (Figure 2). Detailed data on the 8 HGBL cases are provided in Table III.

Treatment and outcome

Detailed treatment information was unavailable for 36 cases (52.2%). The combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) was the most common approach in the cases with available data

Table II. Microscopic features, immunohistochemical findings, EBV status, and molecular findings of the 69 cases of DLBCL and HGBL investigated in this study

Microscopic variables	*N = 69*	*Total (%)*
Bcl2 expression		
Positive	47	68.1
Negative	22	31.9
c-Myc expression		
Positive	22	31.9
Negative	33	47.8
ND	14	20.3
CD10 expression		
Positive	25	36.2
Negative	44	63.8
Bcl6 expression		
Positive	51	73.9
Negative	18	26.1
MUM1 expression		
Positive	44	63.8
Negative	25	36.2
Ki67 index		
Low	34	49.3
High	35	50.7
Bcl2/c-Myc co-expression		
Positive	2	2.9
Negative	53	76.8
ND	14	20.3
Hans algorithm		
GCB	40	58.0
Non-GBC	29	42.0
Muris et al. algorithm		
Favorable prognosis	49	71.0
Unfavorable prognosis	20	29.0
EBV status		
Positive	3	4.3
Negative	66	95.7
BCL2 translocation		
Positive	11	15.9
Negative	34	49.3
ND	24	34.8
BCL6 translocation		
Positive	5	7.2
Negative	40	58.0
ND	24	34.8
MYC translocation		
Positive	11	15.9
Negative	34	49.3
ND	24	34.8
BCL2/BCL6 translocations		
Positive	0	0
Negative	45	65.2
ND	24	34.8
MYC/BCL2 translocations		
Positive	3	4.3
Negative	42	60.9
ND	24	34.8
MYC/BCL6 translocations		
Positive	2	2.9
Negative	43	62.3
ND	24	34.8
MYC/BCL2/BCL6 translocations		
Positive	3	4.3
Negative	42	60.9
ND	24	34.8
Predominant cell type		
Centroblast	50	72.5
Immunoblast	19	27.5
Starry sky pattern		
Presence	18	26.1
Absence	51	73.9
Necrosis		
Presence	63	91.3
Absence	6	8.7
Mitotic ratio (mean: 4.4 mitoses/HPF)		
<4.4 mitoses/HPF	41	59.4
>4.4 mitoses/HPF	28	40.6

EBV, Epstein-Barr virus; *DLBCL*, diffuse large B-cell lymphoma; *HGBL*, high-grade B-cell lymphoma; *ND*, information not described; *GCB*, germinal center B-cell type; *non-GBC*, activated B-cell type; *HFP*, high-power field.

(13 cases), and 5 patients received the CHOP scheme combined with rituximab (R-CHOP). Fifteen cases were submitted to other treatments, including the combined use of surgery, radiotherapy, and other chemotherapy approaches. Survival status was available for 41 patients. Nine Nineteen patients were alive at their last follow-up (27.5%), and 22 patients were dead (31.9%; Table I). The mean follow-up time available for 33 cases was 24 months, ranging from 1 to 83 months. HGBL status was only available for 1 patient (with both *MYC/BCL6* rearrangements). This patient was alive after 12 months of follow-up, with no treatment data available.

Analyses of prognostic parameters

Cross-tabulation analyses revealed that patients who presented ulcerated neoplasms (P = .01) and those tumors that microscopically exhibited evident areas of tissue necrosis were significantly associated with death (P = .05). However, none of the immunohistochemical markers, the EBV status, and the fluorescence ISH findings showed any significant association with patients' status at last follow-up (Supplemental Table S1-S5; available at [URL/link]). However, we also observed that cases expressing c-Myc were significantly associated with a higher proliferative index measured by Ki67 expression (P = .006), as well as those cases exhibiting *BCL2* and *MYC* translocations (both P = .02), as demonstrated in Supplemental Table S1-S5 (available at [URL/link]).

The overall survival rate of our sample was 48.9% and 44.4% after 2 and 5 years of follow-up, respectively (Figure 3). Using log-rank univariate analysis, we observed that females and symptomatic patients were

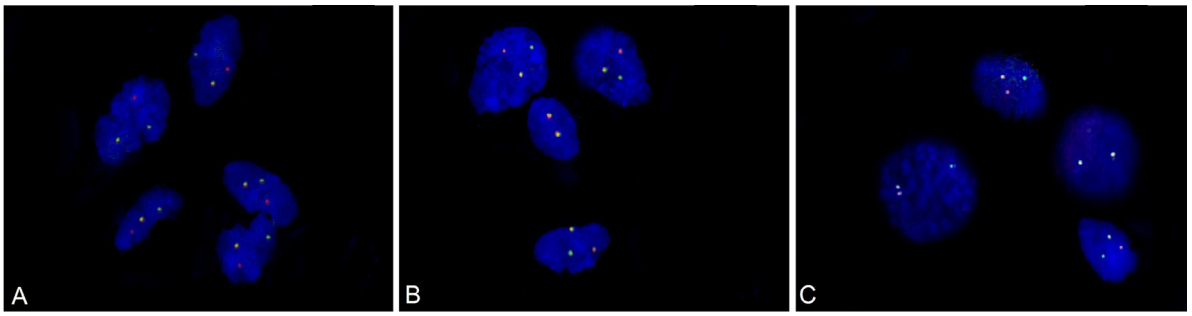

Fig. 2. Fluorescence in situ hybridization findings. A case reclassified as triple-hit HGBL, with translocation of **(A)** *MYC*, **(B)** *BCL2*, and **(C)** *BCL6* genes.

significantly associated with a lower survival rate ($P = .05$ and $P = .01$, respectively), as well as tumors with a microscopic "starry sky" appearance ($P = .03$), co-expression of Bcl2/c-Myc proteins ($P = .006$), and single translocation of the *MYC* gene ($P = .05$; Suplemental Table S1-S5 , available at [URL/link]; Figure 3). It was not possible to investigate the prognostic significance of double-hit and triple-hit HGBL because of the small number of cases. Although we did not find a significant association between the treatment modality and patients' survival rate in the univariate analysis, we included this variable in our Cox regression model because of its importance in the patients' prognosis. Nevertheless, none of the variables included retained their statistical significance and therefore could not be considered independent determinants of lower survival for the evaluated patients ($P > .05$). Finally, we have also carried out cross-tabulation and survival analyses excluding all double-hit and triple-hit HGBL (8 cases) and EBV-positive DLBCL, NOS (3 cases) from the sample, but no difference in the results was observed. However, the remaining DLBCL, NOS presented a slightly inferior survival rate at 5-year follow-up (40%).

DISCUSSION

Clinicopathological information regarding oral and oropharyngeal DLBCL, NOS is currently limited, precluding a better understanding of its biological behavior.[15-21] In addition, there are only a few case reports on the recently described HGBL affecting these sites.[11,12] Therefore, we investigated the clinicopathologic and prognostic features of these tumors and we observed that a very small subset of DLBCLs could be reclassified as either double-hit or triple-hit HGBL. Moreover, the high expression of Ki67 was significantly associated with the presence of *MYC* and *BCL2* rearrangements. Although we did not find any independent prognostic factors, the co-expression of Bcl2/c-Myc proteins and the single translocation of *MYC* may be important determinants in the patient's survival.

The lymphoid tissues that comprise the Waldeyer's ring are the most frequently involved extranodal sites in DLBCL, NOS in the head and neck,[22,23] whereas in the oral cavity the gingiva and hard palate are the most commonly affected locations.[8,18,20,24] In comparison with nodal locations, the clinicopathologic features of extranodal DLBCL, NOS are usually not significantly different.[24] However, some authors have demonstrated that survival rates might vary depending on the primary site affected.[18,23-25]

Oral/oropharyngeal DLBCL, NOS is a rapidly growing neoplasm, presenting a variable clinical appearance. We observed that the presence of pain and ulceration were significantly associated with a lower survival rate, which has not been previously described.[21,26] Given that the presence of symptoms depends on the site of extranodal involvement, the local manifestation of pain may represent a characteristic of oral/oropharyngeal DLBCL, NOS, particularly in cases with ulceration, not necessarily present in nodal or other extranodal DLBCL, NOS.

DLBCL cases may be classified as GCB type or non-GCB type based on their gene expression profile, which is considered a prognostic tool for these patients. However, given the very low availability of this laboratorial analysis in the daily pathology workflow, Hans et al.[13] attempted to apply this approach on an immunohistochemical basis, recommending the analysis of CD10, Bcl6, and MUM1 expressions to categorize the tumors as either GCB or non-GCB group, which is believed to have some prognostic importance for patients treated with the R-CHOP chemotherapy regimen.[5] On the other hand, Muris et al.[14] indicated the use of CD10, Bcl2, and MUM1 to stratify cases as having a favorable or unfavorable prognosis. It was previously shown that GCB DLBCL, NOS is more frequent than the non-GCB subgroup, which is in accordance with our study.[25,27] Regarding the prognostic importance of these algorithms, the non-GCB subtype has been associated with a worse outcome in both nodal and extranodal sites,[28] although Sato et al.[18] observed a high survival rate in patients with non-GBC DLBCL, NOS involving the oral cavity. When the algorithms were compared, the Hans et al. classification presented a significantly better prognostic value.[13,14,29,30] We did not find any significant prognostic potential for either algorithms, as also described by other

Table III. Demographics, clinicopathological features, microscopic and immunohistochemical findings, and EBV status of the 8 HGBL cases investigated in this study

Variables	*Double-hit HGBL*					*Triple-hit HGBL*		
	MYC/BCL2 *translocations*			MYC/BCL6 *translocations*		MYC/BCL2/BCL6 *translocations*		
	Case 1	*Case 2*	*Case 3*	*Case 4*	*Case 5*	*Case 6*	*Case 7*	*Case 8*
Age (years)	75	65	67	72	73	33	72	58
Sex	Female	Male	Male	Female	Female	Male	Male	Female
Site	Oropharynx	Oral cavity	Oropharynx	Oral cavity	Oral cavity	Oral cavity	Oral cavity	Oropharynx
EBV status	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Ki67 index (%)	78.5	90.0	90.0	70.0	90.0	70.5	70.0	90.0
Cell of origin	GCB	GCB	GCB	Non-GBC	GCB	Non-GBC	Non-GBC	Non-GBC
Muris et al. algorithm	Favorable	Favorable	Favorable	Unfavorable	Favorable	Unfavorable	Favorable	Unfavorable
Predominant cell type	Immunoblast	Centroblast	Centroblast	Immunoblast	Centroblast	Centroblast	Immunoblast	Centroblast
Starry sky pattern	Absence	Presence	Absence	Absence	Presence	Presence	Absence	Absence
Mitotic rate (mitoses/HPF)	1.6	4.6	13.4	5.4	5.4	4.0	5.6	2.6
Bcl2 expression	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Positive
CD10 expression	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Negative
Bcl6 expression	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Positive
MUM1 expression	Positive	Negative	Negative	Positive	Positive	Positive	Negative	Positive
c-Myc expression	Positive	Positive	Positive	Negative	Positive	Negative	Positive	Positive

EBV, Epstein-Barr virus; *HGBL*, high-grade B-cell lymphoma; *GCB*, germinal center B-cell type; *non-GBC*, activated B-cell type; *HFP*, high-power field.

authors, suggesting that they may not be reliable and reproducible instruments to stratify patients according to their risk of death.[13,31-34]

The so-called double-expressor lymphoma presents co-expression of c-Myc and Bcl2, and accounts for approximately one-third of de novo DLBCLs.[6,35] According to our results, double-expressor lymphoma was associated with a worse outcome in the univariate analysis, although it lost significance in the multivariate model, possibly because of our relatively small sample size. Previous studies have also demonstrated the prognostic relevance of the simultaneous expression of c-Myc and Bcl2 proteins in DLBCL.[36-38]

According to the literature, approximately 5% to 18% of DLBCL, NOS cases harbor a *MYC* gene translocation for both nodal and extranodal sites,[36,39,40] though we found this translocation in 24.4% of our oral/oropharyngeal cases. The presence of concurrent *BCL2* and/or *BCL6* translocations is lower, comprising about 5% to 15% of cases, which are now classified as HGBL.[35,41,42] As recently described, we also observed that single *MYC* rearrangement was significantly associated with a lower overall survival.[36] Despite recommended as a screening algorithm, the microscopic evaluation ("starry sky pattern"), the expression of c-Myc, Bcl2, and/or Bcl6 proteins, as well as the cell of origin categorization do not reliably indicate the diagnosis of HGBL.[10,39,43,44] Although we observed that a high proliferative index using Ki67 was significantly associated with the occurrence of single *MYC* and *BCL2* translocations, such correlations were not described in previous studies.[45-47] Therefore, the search for immunohistochemical markers that could indicate the diagnosis of HGBL is still desirable to facilitate the diagnostic workflow of this new and aggressive entity, especially in developing countries.

EBV-positive DLBCL, NOS comprises 5% to 15% of all DLBCLs, frequently affecting males over age 50, although sporadic cases are also reported in younger patients,[48] as observed in our sample. Although immunohistochemistry may be used to detect EBV in DLBCL NOS, ISH is the most sensitive method, with more than 80% of atypical cells being positive for the virus.[48-50] The prognosis of EBV-positive DLBCL, NOS differs significantly depending on the patient's . With a cutoff value of 45 years, younger patients are likely to present a better prognosis in comparison with older individuals.[51-54] We were unable to assess the role of EBV in patient outcomes because of the small number of EBV-positive cases in our sample. Nevertheless, a 23-year-old patient was alive after 29 months of follow-up, whereas a 54-year-old patient died less than 1 month after diagnosis. A 29-year-old patient was lost to follow-up.

Currently, the standard therapy for patients with DLBCL, NOS is R-CHOP, which is associated with 60%

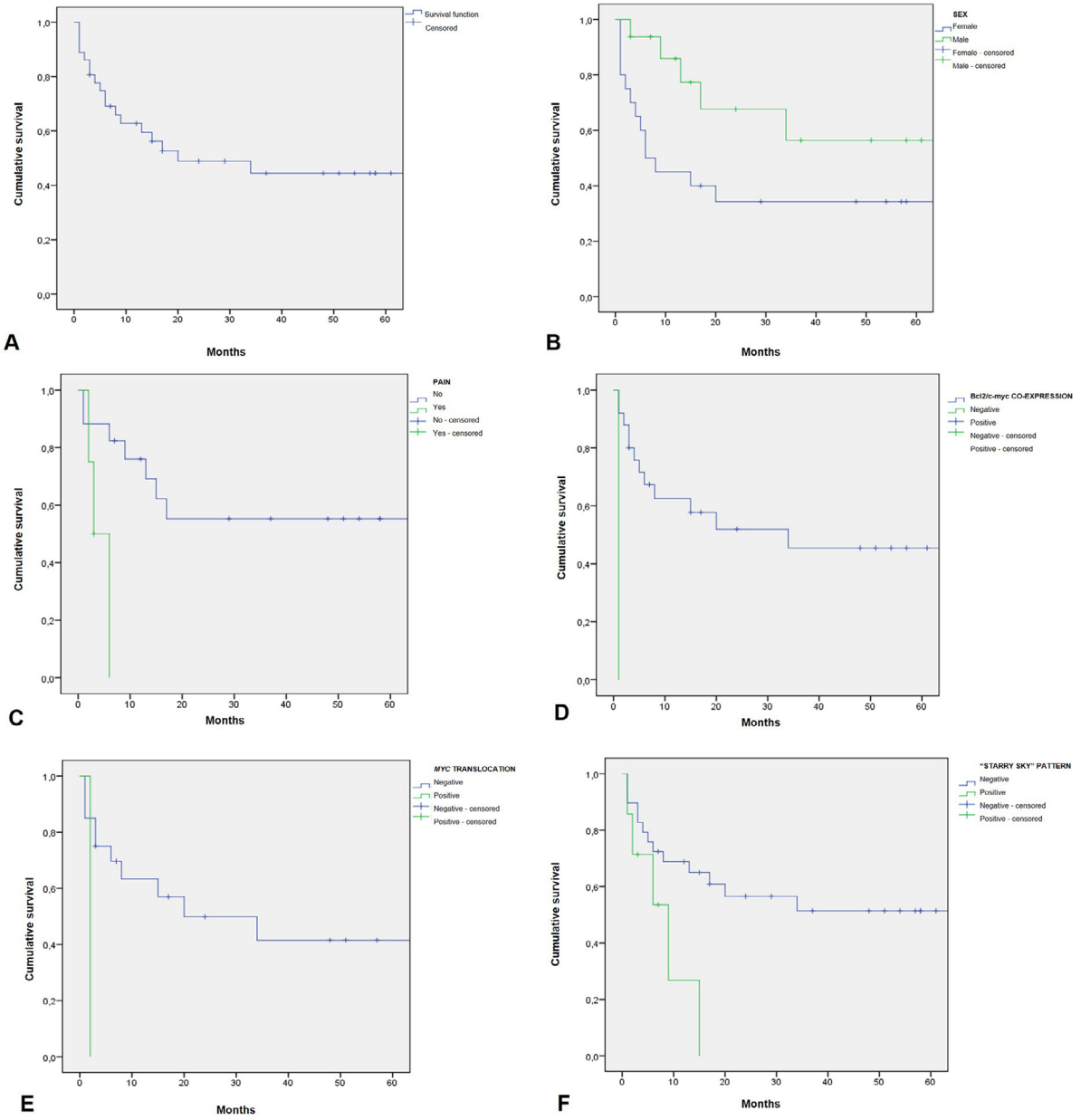

Fig. 3. Kaplan-Meier curves obtained after after log-rank univariate analysis. **(A)** Overall patient survival. **(B)**-**(F)** Parameters that significantly affected patients' survival, using log-rank univariate analysis: **(B)** sex ($P = .05$), **(C)** pain ($P = .01$), **(D)** Bcl2/c-Myc co-expression ($P = .006$), **(E)** *MYC* translocation ($P = .05$), and **(F)** "starry sky" microscopic pattern ($P = .03$).

to 65% of cured cases, and an overall survival of about 50% after 5 years of follow-up for nodal and extranodal cases.[1,55,56] Because of the small number of cases treated with R-CHOP with available follow-up data, we could not investigate whether patients affected by oral/oropharyngeal DLBCL, NOS would significantly benefit from this scheme when compared to CHOP therapy.

Previous studies reported the overall 5-year survival rates for oral and oropharyngeal DLBCL, NOS of 45% and 84%, respectively.[21,57] In our recent review of oral DLBCL, NOS, we observed a 5-year survival rate of 83%,[8] higher than our current sample (44.4%). When we removed the HGBL and the EBV-associated DLBCL, NOS cases from the sample, this survival rate decreased to 40%. These results may be related to patients in our sample who did not undergo treatment because of health care constraints, which may have negatively affected the survival rate. Additionally, we observed that a large number of cases reported in literature lacked appropriate immunohistochemical investigation to confirm the DLBCL, NOS diagnosis. Therefore, some cases included in our previous literature review could represent other less aggressive B-cell lymphomas, likely leading to a higher survival rate. Unfortunately, the small number of

HGBL and EBV-associated DLBCL, NOS did not allow us to determine the specific survival rates.

In an attempt to overcome the paucity of oral and oropharyngeal lymphomas, we collected cases from different pathology institutes; however, such approach may potentially affect our survival results due to possible heterogeneities in the therapeutic schemes used by each center. Furthermore, for future contributions, access to additional clinical data regarding systemic manifestations, B symptoms, and International Prognostic Index values, as well as a more extended follow-up period would strengthen the results.

In conclusion, this study demonstrated the low survival rate among Brazilian patients affected by oral and oropharyngeal DLBCL and that double/triple-hit HGBL and EBV−positive DLBCL, NOS account for a small number of cases previously diagnosed as DLBCL, NOS in these anatomic sites.

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Table S1. Set of antibodies used in this study.

Antibody	*Manufacturer*	*Clone*	*Dilution*	*Antigen retrieval*	*Positive control*
LCA	Dako, Carpinteria, CA, USA	2B11+PD7/26	1:200	Citrate buffer (pH 6.0)	Tonsil
CD3	Dako, Carpinteria, CA, USA	F7.2.38	1:100	Citrate buffer (pH 6.0)	Tonsil
CD10	Dako, Carpinteria, CA, USA	56C6	1:100	EDTA/TRIS (pH 9.0)	Tonsil
CD20	Dako, Carpinteria, CA, USA	L 26(1,2)	1:300	Citrate buffer (pH 6.0)	Tonsil
Cyclin D1	Dako, Carpinteria, CA, USA	DCS-6	1:100	EDTA/TRIS (pH 9.0)	Kaposi sarcoma
Bcl-2	Dako, Carpinteria, CA, USA	124	1:50	Citrate buffer (pH 6.0)	Lymph node
Bcl-6	Santa Cruz, Santa Cruz, CA, USA	PG-B6p	1:300	EDTA/TRIS (pH 9.0)	Tonsil
MUM1	Dako, Carpinteria, CA, USA	MUM1p	1:500	EDTA/TRIS (pH 9.0)	Tonsil
c-myc	Abcam, Cambridge, UK	Y69	1:100	EDTA/TRIS (pH 9.0)	Burkitt lymphoma
Ki67	Dako, Carpinteria, CA, USA	MIB-1	1:100	EDTA/TRIS (pH 9.0)	Squamous cell carcinoma
AE1/AE3	Dako, Carpinteria, CA, USA	AE1/AE3	1:300	Citrate buffer (pH 6.0)	Fibrous hyperplasia
Vimentin	Dako, Carpinteria, CA, USA	Vim 3B4	1:400	Citrate buffer (pH 6.0)	Uterus
Plasma cell	Dako, Carpinteria, CA, USA	Vs38c	1:400	Citrate buffer (pH 6.0)	Tonsil
CD138	Dako, Carpinteria, CA, USA	MI 15	1:100	Citrate buffer (pH 6.0)	Fibrous hyperplasia
CD79a	Dako, Carpinteria, CA, USA	JCB 117	1:1000	Citrate buffer (pH 6.0)	Tonsil
PAX5	Novocastra, Newcastle, UK	1EW	1:50	EDTA/TRIS (pH 9.0)	Tonsil
TdT	Dako, Carpinteria, CA, USA	Policlonal	1:50	EDTA/TRIS (pH 9.0)	Lymphoblastic lymphoma
CD45RO	Dako, Carpinteria, CA, USA	UCHL 1	1:200	Citrate buffer (pH 6.0)	Tonsil
CD56	Novocastra, Newcastle, UK	1B6	1:50	EDTA/TRIS (pH 9.0)	Intestine
CD68	Dako, Carpinteria, CA, USA	KP-1	1:300	Citrate buffer (pH 6.0)	Mucocele
CD30	Dako, Carpinteria, CA, USA	Ber-H2	1:500	EDTA/TRIS (pH 9.0)	Tonsil
CD43	Dako, Carpinteria, CA, USA	DF-T1	1:200	EDTA/TRIS (pH 9.0)	Tonsil
CD5	Dako, Carpinteria, CA, USA	CD5/54/F6	1:300	EDTA/TRIS (pH 9.0)	Tonsil

Table S2. Association analysis of the clinicopathological features, microscopic, immunohistochemical, molecular findings, and EBV-status with the status of the patients affected by oral/oropharyneal DLBCL and HGBL at their last follow-up.

Variables	*Alive N* (%)	*Dead N* (%)	p-*value*
Sex			
Male	11 (57.9)	8 (36.3)	0.17
Female	8 (42.1)	14 (63.6)	
Age (mean age: 60 yrs)			
< 60 yrs	7 (36.8)	9 (40.9)	0.79
> 60 yrs	12 (63.2)	13 (59.0)	
Site			
Oral cavity	15 (78.9)	15 (68.2)	0.44
Oropharynx	4 (21.1)	7 (31.8)	
Symptoms			
Pain	1 (8.3)	4 (36.4)	0.10
Asymptomatic	11 (91.7)	7 (63.6)	
Swelling			
Presence	16 (18.8)	14 (42.9)	0.29
Absence	3 (81.2)	6 (57.1)	
Ulcer			
Presence	6 (33.3)	11 (38.9)	0.01
Absence	12 (66.6)	7 (61.1)	
Bleeding			
Presence	1 (12.5)	0 (0)	0.30
Absence	7 (87.5)	8 (100)	
Bone destruction			
Presence	2 (22.2)	1 (16.6)	0.79
Absence	7 (77.8)	5 (83.3)	
Treatment			
CHOP	6 (35.3)	7 (46.6)	0.42
R-CHOP	4 (23.5)	1 (6.7)	
Others*	7 (41.1)	7 (46.6)	
Bcl2 expression			
Positive	13(31.6)	14 (36.3)	0.75
Negative	6 (68.4)	8 (63.6)	
c-myc expression			
Positive	4 (28.6)	8 (47.0)	0.29
Negative	10 (71.4)	9 (52.9)	
CD10 expression			
Positive	7 (36.8)	5 (22.7)	0.32
Negative	12 (63.2)	17 (77.2)	
Bcl6 expression			
Positive	15 (78.9)	18 (81.8)	0.82
Negative	4 (21.0)	4 (18.2)	
MUM1 expression			
Positive	13 (68.4)	15 (68.2)	0.99
Negative	6 (31.6)	7 (31.8)	
Ki67 index			
Low	10 (47.4)	13 (40.9)	0.68
High	9 (52.6)	9 (59.0)	
Bcl2/c-myc co-expression			
Positive	0 (0)	1 (5.9)	0.36
Negative	14 (100)	16 (94.1)	
Cell of origin			
GCB	11 (57.9)	10 (45.5)	0.43
Non-GBC	8 (42.1)	12 (54.5)	
Muris et al. algorithm			
Favourable	13 (68.4)	15 (68.1)	0.99
Unfavourable	6 (31.6)	7 (31.8)	
EBV status			
Positive	1 (5.3)	1 (4.5)	0.92
Negative	18 (94.7)	21 (95.5)	
BCL2 translocation			
Positive	3 (27.3)	1 (7.1)	0.17
Negative	8 (72.7)	13 (92.9)	
BCL6 translocation			
Positive	1 (9.1)	0 (0)	0.25
Negative	10 (90.9)	14 (100)	
MYC translocation			
Positive	1 (9.1)	1 (7.1)	0.86
Negative	10 (90.9)	13 (92.9)	
MYC/BCL6 translocations			
Positive	1 (9.1)	0 (0)	0.25
Negative	10 (90.9)	14 (100)	
Predominant cell type			
Centroblast	17 (89.5)	16 (27.3)	0.18
Immunoblast	2 (10.5)	6 (72.7)	
Starry sky pattern			
Presence	3 (15.8)	7 (31.8)	0.23
Absence	16 (84.2)	15 (68.2)	
Necrosis			
Presence	16 (84.2)	22 (100)	0.05
Absence	3 (15.8)	0 (0)	
Mitotic ratio (mean: 4.4 mit/HPF)			
< 4.4 mit/HPF	10 (52.6)	15 (68.2)	0.31
> 4.4 mit/HPF	9 (47.4)	7 (31.8)	

*Cases that neither specified the chemotherapy scheme, or did not perform any treatment. GCB: Germinal centre B-cell type; Non-GBC: Activated B-cell type. HPF: high power field.

Table S3. Association analysis of immunohistochemical and molecular findings with Ki67 expression.

Variables	*Ki67 low N* (%)	*Ki67 high N* (%)	p-*value*
Bcl2 expression			
Positive	23 (67.6)	24 (68.6)	0.93
Negative	11 (32.4)	11 (31.4)	
Bcl6 expression			
Positive	23 (67.6)	28 (80.0)	0.24
Negative	11 (32.4)	7 (20.0)	
c-myc expression			
Positive	5 (20.0)	17 (56.7)	0.006
Negative	20 (80.0)	13 (43.3)	
Bcl2/c-myc co-expression			
Positive	1 (4.0)	1 (3.3)	0.90
Negative	24 (96.0)	29 (96.7)	
Cell of origin			
GCB	22 (64.7)	18 (51.4)	0.26
Non-GBC	12 (35.3)	17 (48.6)	

Table S3. Continued

Variables	*Ki67 low N (%)*	*Ki67 high N (%)*	p-*value*
Muris et al. algorithm			
Favourable	25 (73.5)	24 (68.6)	0.65
Unfavourable	9 (26.5)	11 (31.4)	
BCL2 translocation			
Positive	1 (5.9)	10 (35.7)	0.02
Negative	16 (94.1)	18 (64.3)	
BCL6 translocation			
Positive	0 (0)	5 (17.9)	0.07
Negative	17 (100)	23 (82.1)	
MYC translocation			
Positive	1 (5.9)	10 (35.7)	0.02
Negative	16 (94.1)	18 (64.3)	
MYC/BCL2 translocations			
Positive	0 (0)	3 (10.7)	0.16
Negative	17 (100)	25 (89.3)	
MYC/BCL6 translocations			
Positive	0 (0)	2 (7.1)	0.26
Negative	17 (100)	26 (92.9)	
MYC/BCL2/BCL6 translocations			
Positive	0 (0)	3 (10.7)	0.16
Negative	17 (100)	25 (89.3)	

GCB: Germinal centre B-cell type; non-GBC: Activated B-cell type.

Table S4. Log-rank univariate analysis of the clinicopathological features and microscopic findings in the current sample of oral and oropharyngeal DLBCL and HGBL cases.

Clinicopathological variables	*Log-rank univariate analysis*			
	5-years survival (%)	*Estimative (95% CI)*	*Chi-square*	p-*value*
Sex				
Male	56.4	54.3 (34.8 – 73.8)	3.90	*0.05*
Female	34.3	26.1 (13.5 – 38.7)		
Age (mean age: 60 yrs)				
< 60 yrs	44.4	34.4 (20 – 48.8)	0.01	*0.91*
> 60 yrs	47.0	43.7 (26.4 – 61.0)		
Site				
Oral cavity	45.9	34.5 (23.9 – 45.1)	0.87	*0.35*
Oropharynx	40.0	35.5 (11.4 – 59.6)		
Symptoms				
Pain	0	4.3 (2.1 – 6.4)	6.33	*0.01*
Asymptomatic	55.3	50 (31.7 – 68.4)		
Swelling				
Presence	52.0	46.6 (31.5 – 61.7)	0.30	*0.58*
Absence	0	21.4 (8.9 – 33.9)		
Ulcer				
Presence	37.3	36.8 (17.8 – 55.9)	1.36	*0.24*
Absence	56.7	38.6 (24.6 – 52.5)		
Bone destruction				
Presence	63.6	46.3 (22.9 – 69.8)	0.08	*0.78*
Absence	66.7	55.5 (34.0 – 77.1)		
Treatment				
CHOP	38.9	28.3 (13.8 – 42.8)	1.28	*0.53*
R-CHOP	80.0	41.6 (25.1 – 58.1)		
Others*	53.8	50.2 (30.5 – 69.9)		
Predominant cell type				
Centroblast	45.9	44.2 (29.9 – 58.5)	1.24	*0.27*
Immunoblast	33.3	21.5 (0.8 – 42.2)		
Starry sky pattern				
Presence	0	7.9 (3.3 – 12.6)	4.53	*0.03*
Absence	51.4	47.6 (33.5 – 61.7)		
Mitotic ratio (mean: 4.4 mit/HPF)				
< 4.4 mit/HPF	39.2	38.2 (22.4 – 53.9)	0.75	*0.39*
> 4.4 mit/HPF	55.5	40.4 (23.9 – 56.9)		

*Cases that neither specified the chemotherapy scheme, or did not perform any treatment. HPF: high power field. The prognostic value of bleeding and necrosis could not be assessed by univariate analysis.

Table S5. Log-rank univariate analysis of the immunohistochemical and molecular findings, and EBV-status in the current sample of oral and oropharyngeal DLBCL and HGBL cases.

Clinicopathological variables	*Log-rank univariate analysis*			
	5-years survival (%)	*Estimative (95% CI)*	*Chi-square*	p-*value*
Bcl2 expression				
Positive	50	39.7 (23.1 – 56.1)	0.10	*0.75*
Negative	41.4	36.9 (20.4 – 53.5)		
c-myc expression				
Positive	17.5	21.7 (5.9 – 37.3)	2.24	*0.13*
Negative	61.1	52.9 (33.9. – 71.9)		
CD10 expression				
Positive	49.1	48.4 (26.5 – 70.3)	0.52	*0.47*
Negative	42.8	30.0 (18.7 – 41.4)		
Bcl6 expression				
Positive	39.1	32.4 (21.5 – 43.3)	0.08	*0.78*
Negative	57.1	47.9 (17.8 – 77.9)		

Table S5. Continued

Clinicopathological variables	*Log-rank univariate analysis*			
	5-years survival (%)	*Estimative (95% CI)*	*Chi-square*	p-*value*
MUM1 expression				
Positive	45.7	43.9 (27.6 – 60.1)	0.10	*0.75*
Negative	41.5	28.5 (14.0 – 43.0)		
Ki67 index				
Low	45.9	43.7 (27.6 – 59.8)	0.12	*0.73*
High	44.9	32.8 (16.0 – 49.6)		
Bcl2/c-myc co-expression				
Positive	0	1 (1 – 1)	7.67	*0.006*
Negative	45.5	43.3 (27.5 – 59.0)		
Cell of origin				
GCB	45.5	44.4 (27.9 – 61.0)	0.51	*0.48*
Non-ABC	44.4	30.2 (15.4 – 45.1)		
Muris et al. algorithm				
Favourable	44.9	42.8 (27.9 – 57.8)	0.09	*0.76*
Unfavourable	42.4	25.1 (10.9 – 39.4)		
EBV status				
Positive	50.0	22.0 (12.3 – 31.7)	0.05	*0.81*
Negative	44.6	42.0 (28.6 – 55.4)		
MYC translocation				
Positive	0	2.0 (2.0 – 2.0)	3.70	*0.05*
Negative	41.5	41.0 (23.3 – 58.6)		

GCB: Germinal centre B-cell type; Non-GBC: Activated B-cell type.