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Original contribution

B-cell non-Hodgkin lymphomas in Uganda: an immunohistochemical appraisal on tissue microarray☆

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Summary The most common non-Hodgkin lymphomas in Uganda are neoplasms of B-cell derivation. The field of B-cell lymphoma immunophenotype has rapidly progressed because of the increasing availability of markers applicable to routine sections. Although the latter have allowed the identification of distinctive lymphoma entities in the developed countries, such approach has not yet been used in Uganda. One hundred twenty-nine formalin-fixed, paraffin-embedded tissue samples from the Department of Pathology of Makerere University were used for tissue micro-array (TMA) construction. Four-micrometer-thick sections were cut from TMAs and stained with hematoxylin and eosin and Giemsa. They were also used for immunohistochemistry and in situ hybridization. According to morphology and immunohistochemistry, lymphoid neoplasms were classified as Burkitt's lymphoma (BL) (95 cases), diffuse large B-cell lymphoma (19 cases), mantle cell lymphoma (4 cases), and B-cell lymphoblastic lymphoma (1 case). In BL, a homogeneous phenotype ($CD10^+$, $Bcl\text{-}6^+$, $Bcl\text{-}2^-$, MUM1/IRF4-, and Ki-67 ~100%) and a stable Epstein-Barr virus integration were found. A distinctive and unusual feature was the frequent plasma cellular differentiation, along with the positivity for CD30 and CD138 (recorded in 35 and 43 cases, respectively). According to our findings, most non-Hodgkin B-cell tumors in Uganda are endemic BLs followed by diffuse large B-cell lymphomas. The rest consist of rare but clinically important entities such as mantle cell lymphoma and B-cell lymphoblastic lymphoma. The availability of TMAs and immunohistochemistry has enabled us to precisely categorize tumors that have so far been diagnosed in Uganda as "high-grade/aggressive" lymphomas on the basis of cell morphology alone.

1. Introduction

Non-Hodgkin lymphomas (NHLs) of B-cell derivation are the most common lymphoid tumors in Uganda. In western countries, these types of tumors are diagnosed not only by using morphology but also other special techniques such as immunohistochemistry, cytogenetics, and molecular genetics, which help pathologists to reach a more accurate diagnosis. The expansion of the above technologies and especially the usage of an increased number of well-defined

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cell markers in the last 2 decades [1] have led to the recognition of new lymphoma entities and the development of highly refined classification criteria [2]. TMA technology enables high-throughput immunohistochemical analysis of protein expression of a large number of specimens on a single slide. Several studies have shown that TMA is very useful in immunohistochemical characterization of malignant lymphomas being substantially representative of the results obtained on whole tissue sections [3,4]. In resource-constrained African countries, including Uganda [5,6], most of the pathological diagnoses are made using light microscopy and only a small panel of antibodies, compared with the ancillary methods used in developed counties, as described above. This not only leads to misdiagnoses, especially with respect to NHLs other than BL, but also notably, the morphological descriptions are made using old terminology such as *lymphosarcoma* and *reticulum cell sarcoma* [7,8]. So far, there has been no study in the literature, based on the systematic application of the World Health Organization (WHO) classification [2] to a large number of African lymphomas. Therefore, the aim of this study was to describe the morphological and immunophenotypic characteristics of B-cell NHLs in Uganda by using TMA technology and a large panel of antibodies.

2. Materials and methods

2.1. Study design and sampling

This cross-sectional descriptive study was carried out in the Department of Pathology Makerere University Medical School, Kampala, Uganda, and the Unit of Hematopathology, Institute of Hematology and Clinical Oncology "L. and A. Seràgnoli," Bologna University School of Medicine, Bologna, Italy. Six hundred formalin-fixed, paraffin-embedded biopsies were retrieved from the Registry of the Department of Pathology of Makerere University. They had been diagnosed in the period 1991 to 2000 as NHLs, mostly of the "high-grade" or "aggressive" type. Notably, the fixation time had largely exceeded 48 hours, and embedding had been performed at non–fully controlled temperatures. These biopsies were reevaluated in Bologna by 3 pathologists (S.A.P., L.K.T., and C.C.) on hematoxylin and eosin (H and E)–stained sections. Cases that were excluded from the study were as follows: (1) biopsies which were less than 1 mm in diameter, (2) any biopsy specimens that were considered to be inadequate (ie, poorly preserved, processed, or fixed samples), (3) specimens with extensive tissue necrosis, and (4) cases which were found not to be NHLs. The latter corresponded to 25 cases that represented overt examples of classical Hodgkin lymphoma, nonhematopoietic tumor, or reactive lymphadenitis. Thus, most cases were excluded because of inadequacy of the material examined. After such selection, only 129 remained that were stained with Giemsa, reclassified according to the WHO criteria [2], and used for TMA construction. Age, sex, ethnicity, and tumor primary site were known in most of these cases.

Table 1 Primary antibodies used for the study

Antibody	Clone	Source	Antigen retrieval	Dilution
CD3	SP7	Immunotech	EDTA 750 W	1:250
CD5	54/F6	Dako (Marseille, France)	EDTA 900 W	1:10
CD10	56C6	Novocastra (New Castle, UK)	EDTA 900 W	1:5
CD20	L26	Dako	EDTA 750 W	1:200
CD23	1B12	Novocastra	EDTA 900 W	1:30
CD30	Ber-H2	Prof Falini [a]	EDTA 900 W	1:3
CD38	SPC32	Novocastra	EDTA 750 W	1:10
CD79a	JCB117	Prof Mason [b]	EDTA 750 W	1:10
CD138	–	Neomarkers (Fremont, CA)	EDTA 900 W	1:20
Bcl-1	SP4	Neomarkers	EDTA 900 W	1:20
Bcl-6	PG-B6p	Prof Falini	EDTA 900 W	Undiluted
Bcl-2	124	Prof Mason	EDTA 900 W	1:3
IRTA-1	Mum2EC	Prof Falini	EDTA 900 W	1:2
MUM1/IRF4	–	Prof Falini	EDTA 900 W	1:2
TdT	–	Dako	None	1:30
ALKc	–	Prof Falini	EDTA 900 W	1:2
Ki-67	Mib-1	Dako	EDTA 900 W	1:20

[a] Kindly provided by Prof Brunangelo Falini, Perugia University, Perugia, Italy.
[b] Kindly provided by Prof David Y. Mason, Oxford University, Oxford, UK.

2.2. Tissue microarray construction

The representative tumor regions were identified and marked on the H and E stained slides and subsequently identified on the corresponding tissue blocks. Tissue cylinders of diameter of 1 mm were punched from the marked areas of each block and incorporated into a recipient paraffin block using a precision instrument—the tissue arrayer (Beecher Instruments, Silver Spring, MD). To ensure adequate sampling, each specimen was represented in duplicate using 1-mm cores in the recipient block. Three TMA recipient blocks were made; 2 of these had 48 punches

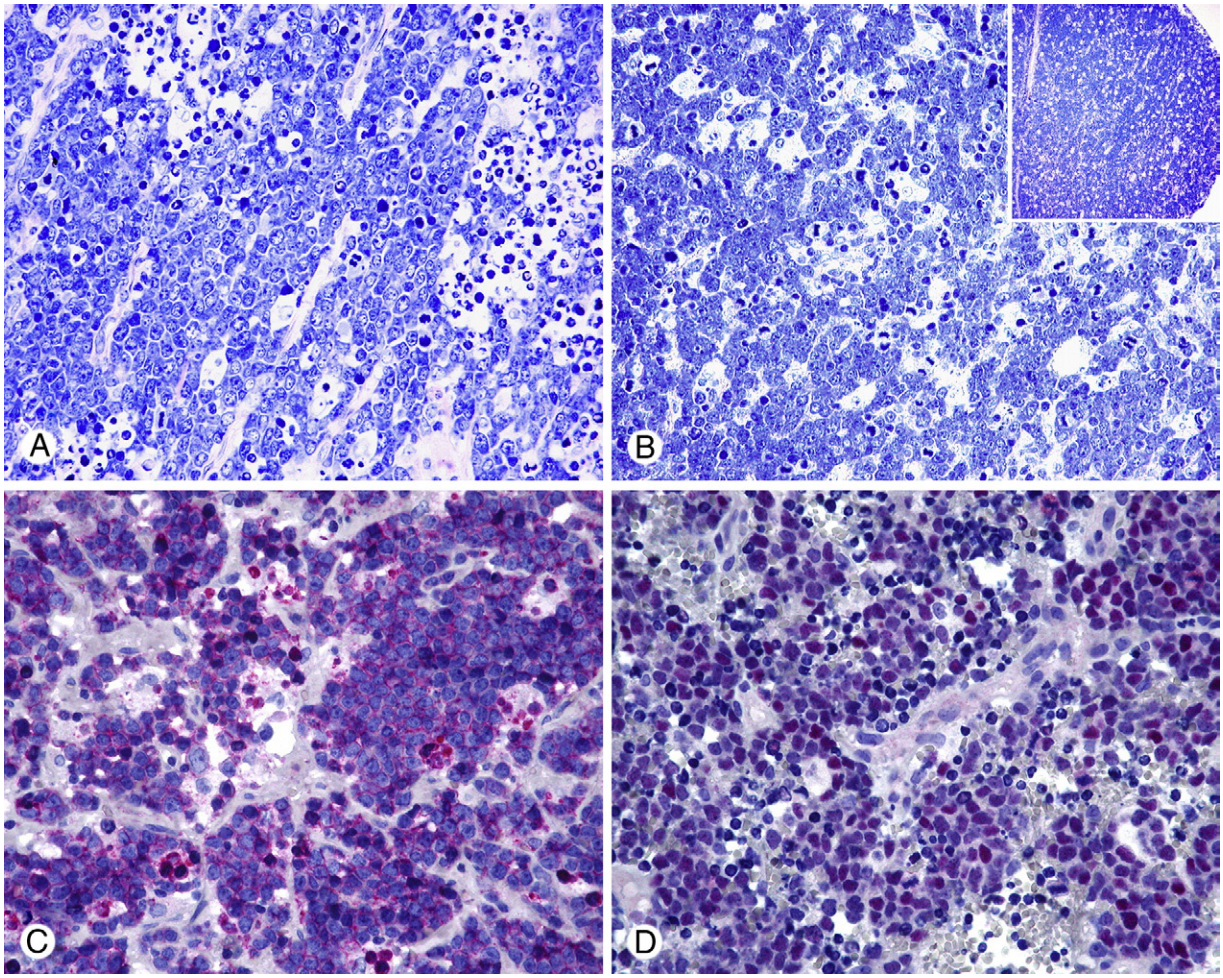

Fig. 1 A, Typical BL (Giemsa staining, original magnification ×40). B, BL with plasmacytoid differentiation (Giemsa staining, original magnification ×40); inset: the same case at low magnification on a Giemsa-stained TMA (original magnification ×2.5). C, CD10 positivity in BL (immunoalkaline phosphatase technique, Gill's hematoxylin counterstaining, original magnification ×40). D, In the same case, Bcl-6 protein expression (immunoalkaline phosphatase technique, Gill's hematoxylin counterstaining, original magnification ×40).

each of Burkitt's lymphoma (BL), whereas the other one had 33 punches of putative NHLs.

2.3. Immunohistochemistry

Four-micrometer-thick sections were cut from TMAs, coated on electrically charged slides, rehydrated, and submitted to antigen retrieval in EDTA 1 mM (pH 8.0) by microwaving twice for 5 minutes at either 750 or 900 W, which proved to be very efficient also in overfixed material according to previous experience [9]. After cooling, the slides were put on a TechMate 500 immunostainer and incubated for 30 minutes at room temperature with antibodies against CD3, CD5, CD10, CD20, CD23, CD30, CD38, CD79a, CD138, Bcl-6, Bcl-2, IRTA-1, MUM1/IRF4, Bcl-1/cyclin D1, TdT, ALKc, and Ki-67/Mib1. Details on the antibodies, sources, dilutions, and antigen retrieval are listed in Table 1. The antibodies were detected by either the alkaline phosphatase anti–alkaline phosphatase immunocomplexes technique or the Envision^{+} technique [10].

2.4. In situ hybridization

2.4.1. Fluorescence in situ hybridization

Four-micrometer-thick sections were cut from blocks and baked in a stove at 56°C. After deparaffination, they were immersed in a 0.2-N HCl solution for 20 minutes. The Paraffin Pretreatment Reagent Kit and the proteolytic solution were then applied at 80°C for 30 minutes and at 37°C for 50 minutes respectively, according to the manufacturer's instructions (Vysis, Abbott Laboratories, Downer's Grove, IL). Hybridization was performed using the Hybrite machine: the slides were co-denaturated with the probe (IGM/MYC/CEP8, Tri-color Dual Fusion Translocation Probe, Vysis, Abbott Laboratories) at 85°C for 5 minutes and then hybridized at 37°C for 22 hours. The slides were washed in 0.4× saline sodium citrate (SSC) at 73°C for 2 minutes and then in 2× SSC at room temperature for 1 minute. Finally, after counterstaining with Diamino-2-phenyl-indole, slides were mounted with a glass cover slip and analyzed with an Olympus BX61 microscope (Olympus, Tokyo, Japan). The

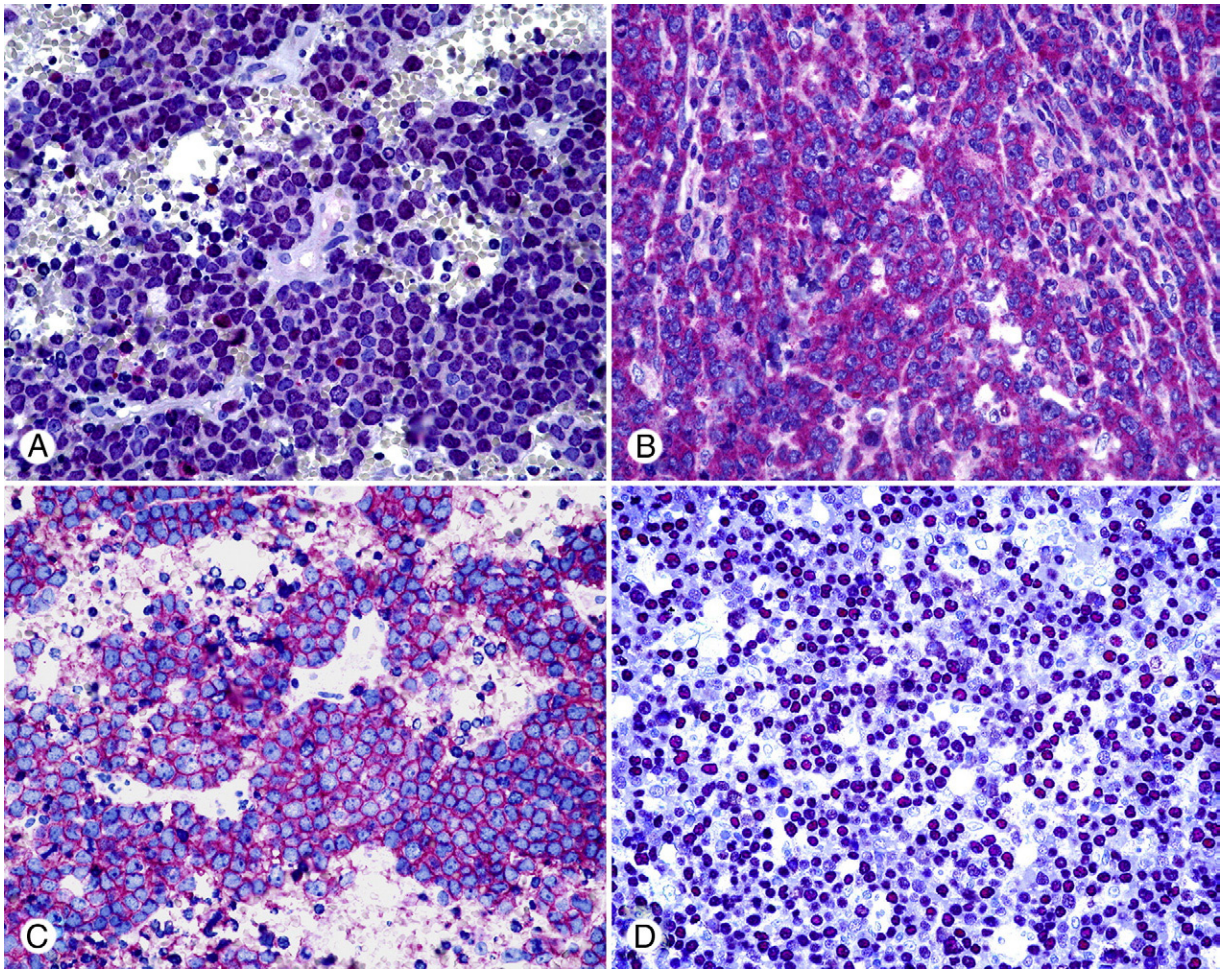

Fig. 2 A, Almost all neoplastic cells of BL do express the Ki-67 antigen as detected by the Mib-1 monoclonal antibody (immunoalkaline phosphatase technique, Gill's hematoxylin counterstaining, original magnification ×40). B, Examples of CD30 positivity in BL (immunoalkaline phosphatase technique, Gill's hematoxylin counterstaining, original magnification ×40). C, the same case as in A shows strong CD138 expression (immunoalkaline phosphatase technique, Gill's hematoxylin counterstaining, original magnification ×40). D, Most if not all BL elements display EBV integration at in situ hybridization (PNA-probe; fast red chromogen; Gill's hematoxylin nuclear counterstaining, original magnification ×40).

efficiency of the probes and system was always assessed by running positive and negative controls in parallel.

2.4.2. Eber

Epstein-Barr virus (EBV) was detected by in situ hybridization using an EBER PNA detection kit (Dako, Glostrup, Denmark) [11] and fast red counterstaining.

2.5. Data management and analysis

Data were collected and entered into the computer using EPI INFO software (supplied by CDC and WHO) for storage and initial analysis. Further analysis was done using SPSS software (SPSS, Chicago, IL) [12]. The data were summarized in frequency tables, graphs, and charts. For continuous variables such as age, the relevant measures of central tendency (means for normally distributed data and medians and interquartile ranges for skewed data) were used to explore the data. Fisher exact test was used for comparison of frequencies. The Mann-Whitney *U* test was used for unpaired comparison of continuous variables. A *P* value of less than .05 was considered significant [13].

3. Results

Of the 129 samples used for TMA construction, 10 cases (8%) were excluded from the study. In particular, 2 cases were reclassified as lymphocyte depleted classic Hodgkin lymphoma and 2 as anaplastic large cell lymphoma after immunohistochemistry. In the other 6 cases, the core biopsy was not representative of neoplastic tissue. The patients' age ranged from 2 to 64 years (median, 16.0; interquartile ranges, 6-20 years). Of 112 cases with sex recorded, 70 (62.5%) were males with a male-to-female ratio of 1.67 to 1. As to ethnicity, Baganda comprised 32 (26.2%); Soga, 12 (9.8%); Teso, 12

(9.8%); Gisu, 8 (6.6%); Lugbara, 6 (4.4%); Nkole, 6 (4.4%); Kiga, 5 (4.1%); Acholi, 4 (3.3%); and Alur, 3 (4.4%). Most were from the capital city Kampala (44, 38.4%); 7 (5.6%), from Masaka, Arua, and Tororo; 6 (4.8%), Mbale; and finally, 4 (3.2%), from Kasese, Luwero, Moroto, and Mukono each. BL was the commonest B-cell tumor (95 cases, 80%), followed by diffuse large B-cell lymphoma (DLBCL) (19 cases, 16%), mantle cell lymphoma (MCL) (4 cases, 3.2%) and B-lymphoblastic lymphoma (1 case, 0.8%). The sites of disease were the abdomen (58 cases, 45%), lymph node (34 cases, 26.3%), and jaw (10 cases, 8.1%). Of the 58 abdominal tumors, 21 (36.2%) were from the ovary; 8 (13.8%), from the intestine; 8 (13.8%), from the mesentery; 2 (3.4%), from the liver; 2 (3.4%), from the kidney; and 1 (1.7%), from the pancreas, the primary site being unspecified in the remaining 10 (17.2%). Morphologically, according to the WHO Classification criteria [2], BL showed a cohesive and monotonous medium-sized neoplastic infiltrate; cells had round nuclei with reticulated chromatin and multiple nucleoli and a narrow rim of basophilic and vacuolated cytoplasm. Mitotic figures were frequent, as well as apoptosis. A typical "starry sky" pattern was present (Fig. 1A). Numerous cases (43/95) exhibited a plasmacytoid differentiation with eccentrically located nuclei and a larger rim of cytoplasm (Fig. 1B). Notably, these cases maintained the chromatin distribution, cytoplasmic basophilia, cohesive growth pattern, abundance of mitotic figures and apoptotic bodies, and "starry sky pattern" seen in typical BL. Most of DLBCLs were immunoblastic lymphomas, characterized by large, oval, irregular, or lobated vesicular nuclei with a prominent central nucleolus and a scanty cytoplasm. Notably, BL always expressed CD10 and Bcl-6 and lacked MUM1/IRF4 (Fig. 1C and D); in all instances but 3, Bcl-2 was negative. Proliferation index turned out to be close to 100% (Fig. 2A). Interestingly, the lymphoid activation molecule CD30 and the plasma cell–associated marker CD138 were found in 35 (37%) and 43 (45%) of the 95 cases, respectively (Fig. 2B and C). Although the former finding was observed irrespective of plasmacytoid differentiation, the latter was strictly associated with plasmacytoid features. EBV integration was detected in all instances (Fig. 2D), except for 12 cases not well preserved, where internal controls were not detected. According to Zinzani et al. [14], DLBCLs were further subdivided into 9 of the activated B cell type ($CD20^+$, $CD10^-$, $Bcl\text{-}6^-$, $Bcl\text{-}2^+$, MUM1/IRF4±, $CD30^+$or $CD138^+$), 5 of the germinal center B-cell type ($CD20^+$, $CD10^+$, $Bcl\text{-}6^+$, MUM1/IRF4±, $Bcl2^-$), and 5 of the unclassified type ($CD20^+$, $CD10^-$, $Bcl\text{-}6^+$, $MUM1/IRF4^+$, $Bcl\text{-}2^+$). Six cases were EBV-positive. MCL had a consistent immune-profile ($CD5^+$, $CD20^+$, Bcl-1/cyclin $D1^+$, $CD10^-$, and MUM1/IRF4-). Of 4 cases, 2 showed a weak and partial positivity for Bcl-6. B-lymphoblastic lymphoma expressed TdT, CD10, and CD20; CD79a was not preserved. Unfortunately fluorescence in situ hybridization analysis turned out not contributory. No signal was in fact observed probably because of tissue overfixation and processing. Statistical analysis did not provide significant results.

4. Discussion

In this article, we describe the morphological and immunophenotypic characteristics of B-cell NHLs in Uganda using the TMA technology.

4.1. Distribution of cases by age, sex, ethnic origin, and tumor site

The results obtained from this study, regarding the age distribution and male-to-female ratio, were compatible to what was previously reported in Central Africa [6,15] where NHLs affect a much younger age group, as compared with the developed countries [16]. Most of the cases were of the Baganda tribe; this is not surprising because it is the main tribe in central Uganda where Mulago Hospital and Makerere University are located. The most common sites affected were the abdomen (45%), then the lymph nodes (26.3%), and lastly, the jaw (8.1%). This is rather surprising because most of the tumors were BL, and it therefore contrasts to what Burkitt [17] found in the 1950s when half of the tumors involved the jaw. However, it is similar to what Cool and Bitter [6] found recently in Kenya. Prospective epidemiologic studies are needed to assess whether the sites of tumor presentation might have changed during the last 50 years in the sub-Saharan area.

4.2. Immunohistochemistry

The lymphomas used for the construction of our TMAs had been previously diagnosed by the Makerere University's pathologists as "high-grade" or "aggressive" NHLs using morphology alone. After immunohistochemistry, they could be classified into distinct entities according to the WHO criteria [2]. This also allowed the identification of the erroneous inclusion of 2 anaplastic large cell lymphoma and lymphocyte depleted classic Hodgkin lymphoma cases respectively, which may be difficult to recognize based on H and E staining only [18]. Notably, the approach applied to this series—that is conventional in western countries but not in Central Africa—is pivotal if one aims to use diversified strategies. In particular, in Uganda, patients with BL, DLBCL, and Hodgkin lymphoma undergo, respectively, COMP (cyclophosphamide, vincristine, methotrexate, prednisone) associated with intrathecal methotrexate prophylaxis, CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone), and MOPP (mechlorethamine, vincristine, procarbazine, prednisone) [19].

4.3. Burkitt's lymphoma

As expected, BL was the most common diagnostic category according to the WHO classification [2]: it accounted for 80% of cases. This result is much higher than what Cool and Bitter [6] found in a similar study in

which NHLs were collected from a diagnostic center serving mission hospitals in Nairobi (Kenya), but lower than that reported by Parkin et al [15] in a case control study in Uganda. This difference might be due to the fact that only a limited number of immunohistochemical markers were used for diagnosis in the aforementioned studies, whereas a large panel of antibodies was used in the present study. In particular, the typical phenotype ($CD10^+$/$Bcl\text{-}6^+$/MUM1/$IRF4^-$, $Bcl\text{-}2^-$, MIb-1 ~100%) [2,5,20] observed in BL cases (irrespectively of plasmacytoid features) supplemented the morphologic details for their distinction from DLBCLs. Remarkably, in a recent study based on highly refined gene expression profiling techniques [21], the combination of immunohistochemistry and cell morphology provided a 90% accuracy in diagnosing BL, MYC fluorescence in situ hybridization adding rather little to this value. Of interest in our series is the high incidence of CD138 and CD30 positive cases (45% and 37%, respectively). The former finding largely corresponded to the plasmacytoid differentiation observed at microscopic evaluation. On the contrary, the latter was recorded both in cases with typical morphology and plasmacytoid features. However, it differs from the prototypic description of Burkitt [17] and, so far, has mainly been reported in western countries among HIV-positive patients [2,22]. The possible influence of HIV infection—affecting 1 000 000 people in Uganda in 2004 [23]—cannot be definitely assessed in our collective because of the lack of serologic information. It is of note, however, that several studies showed that the 3-fold increase of BL recorded in Central Africa during the last decades is independent of the HIV epidemic, most if not all patients being negative for the virus [15,23,24]. Moreover, in general, the prevalence of HIV-related lymphomas is much lower in sub-Saharan Africa than in the west (ie, 20 versus 8000-9000 per 100 000 persons per year), infections being the most common cause of death [23]. In line with this, the most important factor influencing survival of BL patients is still the disease stage [19,25], and only therapies other than COMP might increase the 5-year disease-free survival from the long-established 20% to 25% value [19] to up to 66% in disseminated cases [25].

Certainly, in our series, the CD138 and CD30 positivities might be related to the postulated complex pathogenesis of BL in Central Africa [26]. In particular, Klein [27] proposed a multistep oncogenetic mechanism in which the first stage results in an EBV infection and, the late one, in a de-regulation of *MYC* gene and subsequent development of a malignant clone. In endemic areas, EBV is present in more than 90% of BLs and usually infects very young people promoting immortalization of B-lymphocytes, immune tolerance, and proliferation of infected cells. At the same time, holoendemic malaria has a major role in African BL development: by causing a chronic antigenic stimulation, it increases the number of EBV-positive circulating B-lymphocytes up to 5 times [28] and facilitates cell transformation, especially during acute attacks. On the other hand, malaria inhibits EBV-specific suppressor T cells, and this favors BL through 2 synergistic pathways [29]. The link between malaria and BL is further strengthened by the fact that regional prophylactic malarial programs have shown that where the infection has been eradicated, the cases of lymphoma have decreased. The third cofactor is possibly a plant, *Euphorbia tirucalli*, growing in the African lymphoma belt and recognized as important environmental risk factor for endemic BL. The latex of *E tirucalli* is commonly used by African children in their games; it shows pharmacologic properties as antibacterial, molluscicide, antiherpetic, and antimutagenic agent [30], which explains its wide use in tropical Africa. In vitro, the 4-deoxyphorbol ester, the major biologic mediator in milky latex, seems to be capable of significantly inducing reactivation of EBV lytic cycle gene, reduces EBV-specific T-cells toxicity, and makes B-lymphocytes resistant to cytotoxic T-lymphocytes [31,32].

4.4. Diffuse large B-cell lymphomas

DLBCLs constituted 19 of all the lymphomas tested. By applying the classification criteria proposed by Zinzani et al [14], 9 cases were of activated B cell type, 5 of germinal center B-cell type, and 5 of unclassified type. In 6 cases, EBV was detected. In East Africa, there is limited information about the subclassification of aggressive NHLs other than BL. According to the cancer registry kept in the Department of Pathology, Makerere University Medical School, a dramatic rise in incidence of NHLs has been noted since the advent of the AIDS pandemic in the early 1990s. The behavior of these tumors seems to have changed with more aggressive predominating histotypes, which have a rapid disease progression and very poor outcome [33].

4.5. Mantle cell lymphoma

MCL accounted for 3.2% of the cases tested. Both morphology and immunophenotype ($CD5^+$, $CD20^+$, $BCL1^+$, and $CD10^-$) have enabled us to classify MCL. In particular, one of them was a blastoid variant in agreement with the high proliferation index, while 2 cases showed a weak and partial positivity for Bcl-6 in the absence of CD10 and MUM-1/IRF4 staining, as previously reported by Camacho et al [34]. Unfortunately, the DNA degradation level found in our cases (data not shown) did not allow us to assess whether such positivity was due to Bcl-6 gene aberrations or a high mutational index.

5. Conclusion

This immunohistochemical study based on TMA technology shows that the majority of B-cell NHLs in Uganda

are endemic BLs. Unexpectedly, these reveal rare mandibular location but frequent plasmacytoid differentiation and positivity for CD138 and/or CD30.

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