·Basic Research·

Clinicopathologic features and Epstein-Barr virus infection status of Burkitt's lymphoma in Guangzhou district

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[**Abstract**] **Background and Objective**: Sporadic Burkitt's lymphoma (sBL) is uncommon and its relation to Epstein-Barr virus (EBV) is unknown in China. This study was to investigate the clinical presentation, morphologic features, immunophenotype and EBV infection status of sBL in Guangzhou district, a prevalent area of EBV infection. **Methods**: The clinical data of 21 sBL patients were reviewed. A panel of immunohistochemical staining was performed and EBV-encoded small RNAs (EBERs) in situ hybridization was applied to identify EBV infection. **Results**: From January 2000 to October 2007, 21 cases (0.87%) of sBL were confirmed among 2416 cases of non-Hodgkin's lymphoma (NHL) in Sun Yat-sen University Cancer Center. Male to female ratio was 4.25 (17/4). The median age was 23 years. Of the 21 patients, 19 (90.48%) had lymph node(s) involvement; 16 (76.19%) had multiple sites involvement; 12 (57.14%) were at advanced stages (Ⅲ/Ⅳ). The 2-year survival rate of 15 patients who received chemotherapy or resection plus chemotherapy was 56.00%. Twenty cases showed the prototypic morphology of sBL, and one was the variant of sBL with plasmacytoid differentiation. The main immunophenotype of these 21 sBLs was sIgM$^+$/CD20$^+$/CD10$^+$/Bcl-6$^+$/Bcl-2$^-$[or Bcl-6$^+$(>95%)/Bcl-2$^+$(<10%)]/TdT$^-$/Ki-67$^+$ 100%. Of 20 detectable cases, 11 showed CD5 expression in a few (3%–20%) tumor cells. P53 was overexpressed in ten cases (47.62%). Six cases (28.57%) had EBV infection, with EBNA1 and EBERs expression, but not LMP1. There were no significant differences in morphology and immunophenotype between EBV-positive and EBV-negative cases. **Conclusions**: sBL is uncommon in Guangzhou district, mainly seen in boys and young men. Most patients had lymph node (s) involvement, showing similar morphology and immunophenotype as that of endemic BL. Type I EBV latent infection is associated with 28.57% of cases.

Key words: Burkitt's lymphoma, clinicopathologic features, Epstein-Barr virus, immunophenotype

Burkitt's lymphoma (BL) is an endemic disease in equatorial Africa and Papua New Guinea, and consistently associated with Epstein-Barr virus (EBV) infection. Besides endemic BL (eBL), it may also be seen in immunodeficiency states (immunodeficiency-associated BL) or throughout the world (sporadic BL, sBL). As for sBL, the

frequency is various among different areas of the world. Is it infrequent or frequent in Guangzhou district, a prevalent area of EBV infection in China? This is worthy to be investigated. As compared with the clinicopathologic features of BL described in the new edition of "WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues",[1] are there any characteristics presented in sBL seen in Guangzhou district? It is well known that EBV seroconversion occurs in the first few years of life in Guangzhou population; and Guangzhou district is a high incidence area of EBV-associated nasopharyngeal lymphoid hyperplasia,[2] nasopharyngeal carcinoma (NPC)[3] as well as sinonasal NK/T-cell lymphoma.[4] How many sBLs seen in Guangzhou district are EBV-positive? Is EBV harboring in lymphoid cells of sBL in Guangzhou district the same as that in eBL, also belonging to latent type I? Are there any morphologic and immunophenotypic differences between EBV-positive and EBV-negative sBLs? We investigated 21 cases of sBL by using a panel of immunohistochemical staining and in situ hybridization as well as analysis of clinical data to clarify the issues mentioned above.

1 Materials and Methods

1.1 Materials

A total of 2622 specimens of consecutive lymphomas preserved in the Department of Pathology, Sun Yat-sen University Cancer Center from January 1, 2000 to October 30, 2007 were collected. Among the 2622 cases of lymphoma, 2416 were non-Hodgkin's lymphoma (NHL) and 206 were Hodgkin's lymphoma. According to the clinical presentation, morphology and immunophenotype well defined by the new edition of "WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues",[1] sBL was confirmed in 21 cases. The clinical records of 17 in-patients and four out-patients were reviewed. The formalin-fixed paraffin-embedded blocks of all the biopsies were collected for immunohistochemical staining and EBV-encoded small RNAs (EBERs) in situ hybridization.

1.2 Methods

1.2.1 Immunohistochemical staining The EnVision™ Kit Optimized for DakoCytomaton Automated System (K5007) was used to detect the expression of c-Myc; the Histostain® -Plus kit provided by Zymed Laboratories (Cat. No. 85-9043) was used to detect the other 18 antigens. The pretreatment methods, primary antibodies and their working dilutions used in this study are listed in Table 1.

1.2.2 EBERs in situ hybridization The EBV Probe ISH Kit (NCL-EBV-K) manufactured by Novocastra Company, United Kingdom was used to detect EBERs according to the following steps: (1) deparaffinization and dehydration of the paraffin sections using xylene and a series of graded ethanol; (2) pretreatment with 0.4% pepsin for 7 min; (3) hybridization with FITC-conjugated EBV (EBERs) probe at 37℃ for 16 h; (4)signal detection using alkaline phosphatase-conjugated rabbit F(ab)' anti-FITC and enzyme substrate BCIP/NBT; (5) counterstaining the sections with 1% methyl green solution. The positive signals were blue-black and localized in the nuclei.

2 Results

2.1 Clinical features and laboratory findings

The frequencies of sBL were 0.87% (21/2416) among NHLs and 0.80% (21/2622) among all lymphomas. Of the 21 sBL patients, 17 were Guangzhou district residents, while the residential places of other four out-patients were not clearly recorded; 17 were men and four were women. The patients were aged of 3–72 years, with a median of 23 years. Two patients had extranodal lymphoma (one in the gastric fundus and the other in the ileo-coecal region), nine had lymph node(s) involvement, and ten had both extranodal lymphoma and lymph node(s) involvement. The cervical lymph node(s) (in 17 cases) and inguinal lymph node(s) (in seven cases) were the common sites involved. According to the WHO staging scheme (2001),[5] nine cases were at stage Ⅰ/Ⅱ, and 12 at stage Ⅲ/Ⅳ. The antibody assay against human immunodeficiency virus (HIV) of all the 21 sBL patients was negative. The

Table 1 The manufacturer, pretreatment and working dilution of primary antibodies for detecting antigens used in immunohistochemistry

Manufacturer (Cat. No.)	Pretreatment	Working dilution	Antigen detected
Zymed (18-0367)	HP CB	1:100	CD45
Novocastra (NCL-IgMp)	HP CB	Ready for use	IgM
Novocastra (NCL-IgGp)	HP CB	Ready for use	IgG
Novocastra (NCL-IgAp)	HP CB	Ready for use	IgA
Zymed (18-0192Z)	HP CB	1:50	Ki-67
Zymed (18-0155)	HP CB	1:100	CD20
Novocastra (RTU-CD79α-192)	HP CB	Ready for use	CD79α
Novocastra (RTU-CD3-PS1)	HP CB	Ready for use	CD3
Zymed (18-7237)	HP EDTA	1:50	TdT
Novocastra (RTU-CD10-270)	HP CB	Ready for use	CD10
Zymed (08-1426)	HP EDTA	Ready for use	Bcl-6
Zymed (18-0193Z)	HP CB	1:50	Bcl-2
Zymed (08-1283)	HP CB	Ready for use	CD5
DAKO (M7259)	HP EDTA	1:150	MUM1 (MUM1p)
Zymed (18-7251)	HP CB	1:50	P53 (DO-7)
Chemicon (MAB 8173)	HP EDTA	1:10	EBNA-1
Labvision (MS-1458-R7)	MW EDTA	Ready for use	LMP1 (CS.1-4)
Labvision (MS-1086-R7)	HP CB	Ready for use	CD21
Abcam (ab17767)	HP EDTA	Ready for use	c-Myc (SPM237)

HP CB: boiling with citrate buffer (0.01 mol/L, pH6.0) under high pressure; MW EDTA: microwave with EDTA (1 mmol/L, pH9.0); HP EDTA: boiling with EDTA (1 mmol/L, pH9.0) under high pressure; TdT: terminal deoxynucleotidyl transferase.

lactic acid dehydrogenase (LDH) had been tested in 17 patients, nine of whom showed elevation (278–2864 u/L).

2.2 Morphologic features

With HE staining, the lymphoma cells appeared monotonous and medium-sized, with round nuclei, coarse chromatin, multiple small nucleoli and a small amount of basophilic or amphophilic cytoplasm. The size of the tumor cell nuclei was similar to that of the mixed histiocytes. The lymphoma cells usually exhibited retracted cytoplasm, sometimes contained tiny lipid vacuoles. Tumor cells with a single central nucleolus could be found in some cases. A "starry-sky" pattern was usually created by numerous benign interspersed macrophages that had ingested apoptotic cells or apoptotic bodies (Fig. 1A). Frequent mitotic figures and apoptotic bodies were the constant features. Twenty tumors showed the morphology as described above, being called as the prototype of BL (Fig. 1B). However, a certain degree of pleomorphism in nuclear size and shape of the tumor cells as well as plasmacytoid lymphoid cells were observed in one case (Fig. 1C). The morphology of this tumor was consistent with the diagnosis of BL with plasmacytoid differentiation. The tumor had an extremely high proliferation index and Ki-67 (MIB-1) was detected in nuclei of almost all tumor cells (Fig. 2A). Proliferating blood vessels were found in the tumor parenchyma.

2.3 Immunohistochemical findings

The tumor cells of all 21 sBLs expressed surface membrane IgM (Fig. 2B), but not IgG and IgA. They also expressed CD45, CD20 and CD79α, but not TdT. CD10 was detected on membrane of almost all tumor cells in 21 sBLs (Fig. 2C); however, the staining intensity was varied case from case. As shown in Table 2, the expression of Bcl-6 (Fig. 2D), Bcl-2 and MUM1 found in the 21 sBLs had the following patterns: (1) Bcl-6-positive/Bcl-2-negative/MUM1-negative in five cases; (2)Bcl-6-positive/Bcl-2-positive (<5%)/MUM1-positive (<40%) in eight cases; (3)Bcl-6-positive/Bcl-2-negative/MUM1-positive (<40%) in six cases; (4) Bcl-6-positive/Bcl-2-positive (<10%)/MUM1-negative in two cases. A small

portion of tumor cells (3%–20%) expressed CD5 in 11 of 20 available cases. The expression of c-Myc oncoprotein was seen in all 21 sBLs. c-Myc was expressed mainly in cytoplasm or nuclei (Fig. 2E). A few of P53-positive tumor cells (2%–15%) were found in 11 cases; P53-positive tumor cells with a proportion of over 50% were seen in ten cases, seven of which had P53-positive tumor cells with a proportion of over 80% as shown in Table 3 (Fig. 2F). CD21 was not detected in the 21 sBLs. Only a few CD3-positive reactive T cells were found in the tumor parenchyma of all cases.

2.4 EBV infection status

EBERs signals were detected in lymphoma cell nuclei in six out of 21 sBLs by using in situ hybridization (Fig. 2G). The EBERs-positive tumor

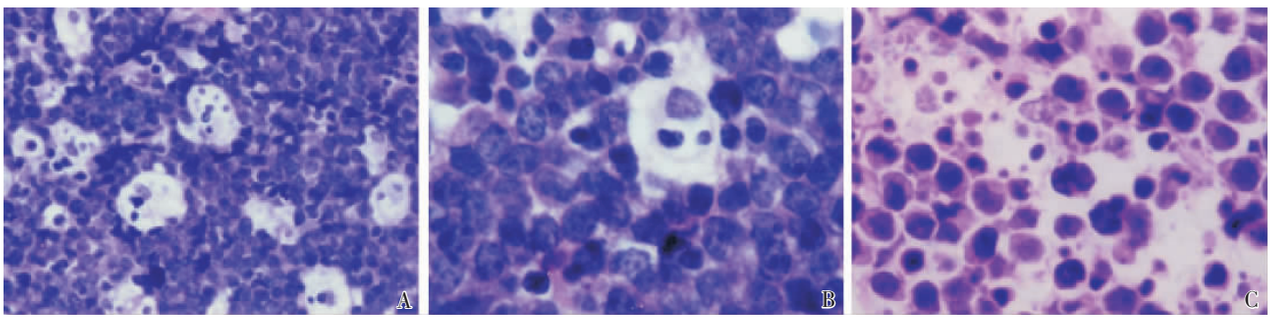

Figure 1 Morphology of Burkitt's lymphoma

A: A "starry-sky" pattern is created by numerous benign interspersed macrophages that had ingested apoptotic cells or apoptotic bodies (HE ×200).

B: The lymphoma cells have a round nucleus with multiple small nucleoli and amphophilic cytoplasm (HE ×500).

C: In BL with plasmacytoid differentiation, neoplastic lymphoid cells with plasma cell appearance are observed (HE ×500).

Table 2 The morphology, Epstein-Barr virus (EBV) detection and antigen expression of the 21 cases of sporadic Burkitt's lymphoma (sBL)

No.	Sex	Age (years)	Histological type	EBERs	EBNA-1	LMP1	CD10	Bcl-6	Bcl-2	MUM1	CD5	P53
1	M	12	Prototype	–	ND	ND	>95%	>95%	5%	40%	–	5%
2	F	30	BLPD	–	ND	ND	>95%	>95%	–	40%	–	50%
3	M	23	Prototype	–	ND	ND	>95%	>95%	–	–	–	2%
4	M	18	Prototype	+	+	–	>95%	>95%	2%	–	10%	10%
5	M	59	Prototype	+	+	–	>95%	>95%	–	–	10%	5%
6	M	20	Prototype	+	+	–	>95%	>95%	–	2%	10%	90%
7	M	3	Prototype	–	ND	ND	>95%	>95%	–	40%	–	100%
8	M	22	Prototype	+	+	–	>95%	>95%	–	–	–	15%
9	M	25	Prototype	–	ND	ND	>95%	>95%	10%	–	ND	15%
10	M	58	Prototype	–	ND	ND	>95%	>95%	10%	5%	10%	3%
11	M	25	Prototype	–	ND	ND	>95%	>95%	–	20%	–	15%
12	M	8	Prototype	–	ND	ND	>95%	>95%	5%	40%	–	10%
13	M	23	Prototype	+	-	–	>95%	>95%	5%	1%	10%	15%
14	F	10	Prototype	–	ND	ND	>95%	>95%	5%	5%	5%	90%
15	M	72	Prototype	–	ND	ND	>95%	>95%	–	5%	10%	50%
16	M	6	Prototype	+	+	–	>95%	>95%	–	–	3%	85%
17	M	36	Prototype	–	ND	ND	>95%	>95%	–	–	–	85%
18	F	16	Prototype	–	ND	ND	>95%	>95%	–	40%	5%	85%
19	M	68	Prototype	–	ND	ND	>95%	>95%	2%	40%	20%	15%
20	M	9	Prototype	–	ND	ND	>95%	>95%	2%	40%	–	85%
21	F	41	Prototype	–	ND	ND	>95%	>95%	5%	10%	5%	50%

M: male; F: female; BLPD: Burkitt's lymphoma with plasmacytoid differentiation; %: percentage of positive tumor cells; +: positive; –: negative; ND: not done.

cells expressed EBV-encoded nuclear antigen-1 (EBNA-1) (Fig. 2H), but not latent membrane protein 1 (LMP1) by using immunohistochemistry (Fig. 2I). Individual EBERs-positive non-neoplastic small lymphocytes were found in three out of 15 EBERs-negative sBLs.

2.5 Follow-up

Fifteen of the 21 sBL patients had been followed up by phone for 1–65 months; six patients were lost. Three patients with LDH elevation died of tumor lysis syndrome during treatment, and three (two of which had LDH elevation) died of sBL at 6–12 months after completing chemotherapy; nine survived for 5 – 65 months after completing chemotherapy (eight cases) or resection plus chemotherapy (one case). The 2-year survival rate was 56.00%.

3 Discussion

As mentioned above, the frequency of sBL is various among different areas of the world. Zhang et al.[6] reported that the frequencies of sBLs were 1.45% (7/482) among NHLs and 1.29% (7/543) among all lymphomas in Beijing. Chang et al.[7] reported that the frequency of sBL was 1.73% (3/173) among all lymphomas in Taiwan, China. The

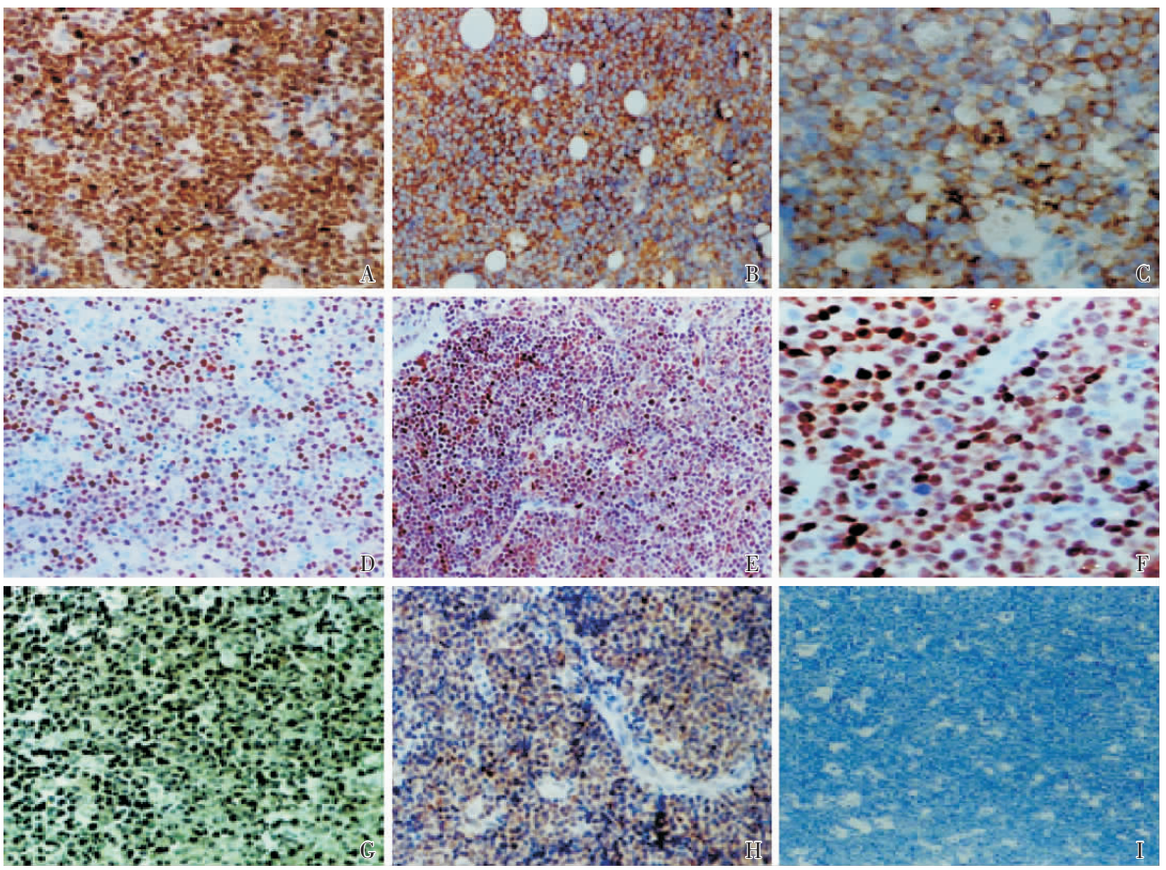

Figure 2 Immunophenotype of Burkitt's lymphoma

A: Almost all lymphoma cells exhibit nuclear Ki-67 expression (IHC ×100).

B: Tumor cells express surface IgM (IHC ×100).

C: The majority of tumor cells express cytoplasmic CD10 with varied intensity (IHC ×200).

D: Almost all lymphoma cells express nuclear Bcl-6 (IHC ×100).

E: Almost all lymphoma cells express nuclear/cytoplasmic c-Myc protein (IHC ×200).

F: The vast majority of tumor cells in this case overexpress P53 protein (IHC ×200).

G: EBERs signals are detected in the nuclei of almost all lymphoid cells (in situ hybridization ×100).

H: The majority of EBV-positive lymphoma cells express EBNA-1 antigen (IHC ×100).

I: The tumor cells of EBV-positive BL with "starry sky" appearance do not express LMP1 (IHC ×50).

frequencies of sBL in Beijing and Taiwan are similar to those in south Korea (1.10% among NHLs and 1.03% among all lymphomas) and Japan (1.15% among NHLs and 1.08% among all lymphomas)[8] as well as those in Western Europe and America (1%–2% among adult lymphomas).[9] Our results show that only 21 cases of sBL were confirmed from January 2000 to October 2007, and the frequency of sBL were 0.87% (21/2416) among NHLs and 0.80% (21/2622) among all lymphomas. Accordingly, the frequency of sBL in Guangzhou is the lowest as compared with the data ever reported in literature so far as we known. However, the frequencies of sBL among NHLs may reach a comparatively high level, such as in Thailand (3.10%)[8] and Jordan (5.51%).[10]

The sBLs in Guangzhou reported herein are more likely to be developed in male children and young adults (male: female = 17/4, median age of 23 years). This coincides with what has been popularly recognized.[1] But we have to point out that no jaw tumor was found in our cases, and lymph node(s) were involved in most cases (19/21). This seems the clinical characteristics of our cases worthy to be mentioned.

In this study, the morphology of 20 sBLs is identical with that of the prototype of BL; the rest one case can be recognized as BL with plasmacytoid differentiation as described by WHO classification.[1] Immunohistochemically, the tumor cells of all 21 sBLs consistently show $CD45^+/CD3^-/CD20^+/CD79\alpha^+/IgM^+/IgG^-/IgA^-/TdT^-/CD10^+$. The combination of antigen expression ($CD45^+/CD3^-/CD20^+/CD79\alpha^+$) implicates that the lymphoma belongs to B-cell lineage, and the restricted expression of surface immunoglobulins ($IgM^+/IgG^-/IgA^-$) can not only illustrate the clonal expansion existed in this tumor but also support the diagnosis of BL. As we known, the expression of IgM is beneficial to differentiate BL from other B-cell lymphomas to some degree. Furthermore, $TdT^-/CD10^+$ is also an immunophenotipic characteristic of BL. In addition, almost all lymphoma cells express nuclear Ki-67, which is also in favor of diagnosing BL.

The antigen expression of Bcl-6, Bcl-2 and MUM1 might be attributed to four patterns being described above. As we known, germinal center cells express Bcl-6, while the lymphoid cells expressing Bcl-2 and/or MUM1 correspond to the activated B cells, that is, though the tumor cells can express activated B-cell markers, such as Bcl-2 and/or MUM1, the percentage of either Bcl-2-positive cells and/or MUM1-positive cells should be lower than that of Bcl-6-positive cells. In brief, a high germinal center cell score or a low activated B-cell score is the critical criterion for diagnosing BL.

It should be pointed out that a small number of neoplastic cells (3%~20%) in 11 out of 20 available cases express CD5, and the tumor cells of five elder patients (> 40-year-old) express CD5. Lin et al.[11] also reported that CD5 expression might be seen in BL cells in elder patients.

It is popularly recognized that most BLs have Myc (also known as c-Myc) translocation.[1] Though the c-Myc translocation can develop in lymphomas other than BL, this kind of gene translocation is still recognized as a fundamental molecular event in pathogenesis of BL.[12] It is a pity that we have not detected the c-Myc translocation in our sBL cases. However, we have performed immunohistochemical staining of c-Myc oncoprotein. As we known, activation of Myc oncogene, which encodes the c-Myc transcription factor, resulting in c-Myc oncoprotein overexpression can mediate lymphomagenesis.[12] Our results show that c-Myc protein was markedly overexpressed in all of the 21 sBLs. This finding once again confirms that c-Myc-mediated oncogenesis plays a key role in the tumorigenesis of sBL in Guangzhou.

If we define $\geqslant 50\%$ of P53-positive lymphoma cells presenting in a sBL tissue as P53 overexpression, the P53 overexpression rate of these 21 sBLs in Guangzhou is 47.62% (10/21). Klumb et al.[13] reported that P53 overexpression rate was positively correlated with p53 gene mutation, and the p53 gene mutation occurred in sBLs may reach up to 33.33% (9/27)[14] or 37.04% (10/27).[15] Accordingly, can we say that the p53 gene mutation rate of sBL in

Guangzhou is somewhat similar to that as reported? A comparative study should be performed between P53 overexpression and p53 gene mutation for sBLs in Guangzhou in the future. Worthy to be noted is that four out of six children sBLs had P53 overexpression, and the percentage of positive cells reached to ≥85%. Does p53 gene mutation more often develop in children sBL? This is also worthy to be further studied.

It is well known that eBL is associated with EBV in over 95% of cases.[16] Endemic EBV-associated BL has an incidence of 5-10/100 000 children and accounts for up to 74% of childhood malignancies in the African equatorial regions.[17] As in sBL, the frequency of EBV association varies with location worldwide. Recently, Kelly et al.[18] said that in North Europe and the US, 15%-20% of BLs were EBV-positive; while in North Africa and North Brazil, 85% of BLs were EBV-positive. Guangzhou district, China is an endemic area of EBV infection. Our results show that the frequency of EBV infection of sBLs in Guangzhou is 28.57% (6/21). This figure is similar to those reported by Chan et al. in Hong Kong (27.78%, 5/18),[19] and Chuang et al. in Taipei, China (25.61%, 8/31).[20] The EBV harboring in lymphoma cells of the six EBV-positive cases expressed EBNA1 and EBERs, and did not express LMP1, implicating that the EBV infection is of type I latency. Worthy to be mentioned is that though the vast majority of eBLs were infected with EBV of type I latency, a variable number of tumor cells of two eBL cases could express LMP1 as reported by Niedobitek et al.[21] LMP1 was really undetectable in our six EBV-positive sBLs seen in Guangzhou. Hence, sBLs may be subclassified into EBV-positive and EBV-negative subtypes. All tumor cells of either EBV-positive or EBV-negative sBLs do not show the EBV receptor CD21 expression. The role of EBV harboring in sBL cells is an issue for further study. Except EBV infection, are there other differences presenting between these two subtypes? Interestingly, recent studies indicate that EBV-positive sBL may derive from memory B cells and EBV-negative sBL from the early centroblasts of lymph-follicle dark zone by studying the mutation rate of V(H) gene and signs of antigen selection.[22,23] However, we cannot find some substantial differences between EBV-positive and EBV-negative sBLs either from clinicopathologic features or from immunophenotypic profile. So, the issue that EBV-positive and EBV-negative sBLs are derived from different cells is worthy of further study.

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