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Diagnostic importance of CD179a/b as markers of precursor B-cell lymphoblastic lymphoma

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Surrogate light chains consisting of VpreB (CD179a) and λ5 (CD179b) are expressed in precursor B cells lacking a complete form of immunoglobulin and are thought to act as substitutes for conventional light chains. Upon differentiation to immature and mature B cells, CD179a/b disappear and are replaced with conventional light chains. Thus, these molecules may be useful as essential markers of precursor B cells. To examine the expression of the surrogate light-chain components CD179a and CD179b in precursor B-cell lymphoblastic lymphoma, we analyzed tissue sections using immunohistochemistry techniques. Among a number of monoclonal antibodies for the surrogate light chains, VpreB8 and SL11 were found to detect CD179a and CD179b, respectively, in acetone-fixed fresh frozen sections. Moreover, we also observed VpreB8 staining in formalin-fixed, paraffin-embedded sections. Using these antibodies, we found that CD179a/b were specifically expressed in precursor B-cell lymphoblastic lymphomas, but not in mature B-cell lymphomas in childhood. Furthermore, other pediatric tumors that must be included in a differential diagnosis of precursor B-cell lymphoblastic lymphoma, including precursor T-cell lymphoblastic lymphoma, extramedullary myeloid tumors, and Ewing sarcoma, were also negative for both CD179a and CD179b. Our data indicate that CD179a and CD179b may be important markers for the immunophenotypic diagnosis of precursor B-cell lymphoblastic lymphomas.
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B cells are characterized by the surface expression of immunoglobulin (Ig), consisting of a heavy chain (HC) and conventional κ or λ light chains (LCs). The Ig expressed in B cells is associated with the Igα/Igβ (CD79a/b) complex and forms a B-cell antigen receptor complex. In contrast to these mature B cells, precursor B cells do not express Ig, although they do contain Ig-related components. For example, more primitive pro-B cells already express the Igα/Igβ complex and contain surrogate LCs, consisting of VpreB (CD179a) and λ5 (CD179b).[1–5] Through the successful rearrangement of HC genes, pro-B cells undergo differentiation into pre-B cells and start to express a premature antigen receptor, namely the pre-B-cell receptor (pre-BCR), consisting of μ HC, CD179a/b and the Igα/Igβ heterodimer.[6–9] Upon further differentiation from pre-B cells to B cells, CD179a/b disappear and are replaced with conventional LC.

The stage-specific developmental expression of Ig-related molecules is an essential characteristic of B-lineage cells and is conserved not only in normal cells but also in neoplastic cells of B lineage. Indeed, precursor B acute lymphoblastic leukemias (ALL), which originate from precursor B cells and lack the complete form of Ig, are known to express CD179a/b, while mature and Ig-expressing B-cell ALLs do not.[10] Precursor B-cell lymphoblastic lymphoma (B-LBL) is a disease in which neoplastic precursor B cells proliferate without the obvious involvement of blood or bone marrow and thus exhibits immunophenotypic characteristics that are similar to those of precursor B-ALL.[11,12] Neoplasms of precursor B cells most commonly present as a form of ALL during childhood, and the presentation of B-LBL is infrequent, but may occur in patients of any

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age, frequently involving the skin, bone, or lymph nodes. Owing to the rareness of B-LBL and its morphological and immunophenotypic similarities to mature B-cell lymphomas in some cases, distinguishing between these diseases is of great importance, especially in the field of pediatric oncology, because the treatment strategies for these two diseases are quite different. In addition, other tumors, including precursor T-cell lymphoblastic lymphoma (T-LBL), extramedullary myeloid tumors, and Ewing sarcoma, must also be included in a differential diagnosis of B-LBL.

In an attempt to characterize B-LBL using the expression of Ig-related molecules and to examine the utility of such a method for diagnosis, we examined CD179a/b expression in B-LBL tissues using immunohistochemistry. CD179a/b was found to be specifically expressed in B-LBL, but not in mature B-cell lymphomas and other tumors in childhood. The usefulness of CD179a/b as diagnostic markers for B-LBL is discussed.

Materials and methods

Materials

The human pre-B-cell line HPB-NULL[10] and the Burkitt cell line Ramos (Japanese Cancer Research Resources Bank, Tokyo, Japan) were used in this study. Cells were maintained in RPMI1640 supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere.

Biopsy specimens from pediatric patients, including 11 patients with B-LBL, seven patients with Burkitt lymphoma, three patients with diffuse large B-cell lymphoma, seven patients with T-LBL, three patients with extramedullary myeloid tumors, and three patients with Ewing sarcoma, were selected from medical files collected between 1985 and 2003 at the Department of Developmental Biology, National Research Institute for Child Health and Development. In each case, the initial diagnosis was based on morphological observations of hematoxylin and eosin (H&E)-stained, formalin-fixed, paraffin-embedded tissues, the immunophenotypic characteristics revealed by immunohistochemistry using acetone-fixed, fresh frozen sections, and the patient's clinical features. In some cases, immunophenotyping was also performed using flow cytometric analysis of a single-cell suspension prepared from the tissue. To examine CD179a/b expression, snap-frozen tissues in OCT compounds stored at −85°C after the initial diagnosis were used.

The following mouse monoclonal antibodies (mAbs) were used in this study: anti-CD179a (HSL96), anti-CD179b (HSL11), anti-conformational pre-BCR (HSL2),[10] anti-CD20 (L26),[13] anti-HLA-DR,[14] and anti-CD10 (IF6).[15] HSL2 is a unique mAb that does not bind to each component of the pre-BCR, but recognizes a conformational epitope formed only when the μ HC and CD179a/b surrogate LC associate with each other to make the pre-BCR complex.[10] In addition, several commercially available mAbs were also used: anti-μ (G20-127), anti-CD179a (VpreB8 and VpreB9), and anti-CD19 (Leu12) from BD Pharmingen (San Diego, CA, USA); anti-κ (HP6053) and anti-λ (HP6054) from Zymed Laboratories Inc. (San Francisco, CA, USA); anti-CD79a (HM-57), anti-CD22 (4KB128), and anti-TdT (HT-1/3/4) from DAKO (Glostrup, Denmark); anti-CD179a (4G7) from Coulter/Immunotech Inc. (Westbrook, MA, USA); anti-TdT (SEN28) from Nichirei Co. (Tokyo, Japan); and anti-CD179a (B-MAD-688) from Biocarta (San Diego, CA, USA). The anti-CD77 (1A4) used in this study was a generous gift from Dr S Hakomori of the University of Washington, Seattle, WA, USA and Otsuka Assay Laboratories, Kawauchi-cho, Tokushima, Japan. Secondary Abs, including fluorescence- and enzyme-conjugated Abs, were purchased from Jackson Laboratory, Inc., West Grove, PA, USA.

Flow Cytometry

The cells were stained with mAbs and analyzed by flow cytometry (EPICS-XL, Coulter) as described previously.[15] Cytoplasmic antigens were stained using CytoStain™ Kits (BD Pharmingen), according to the manufacturer's protocol.

Immunohistochemistry

Immunohistochemical staining of acetone-fixed fresh frozen sections was performed as described elsewhere.[16] Briefly, fresh frozen sections from each tissue were prepared using a cryostat apparatus and fixed in acetone for 15 min at 4°C. After washing in phosphate-buffered saline (PBS) and blocking with normal rabbit serum, the sections were incubated with mAbs at appropriate dilutions for 30 min at room temperature. Sections were then washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibodies for 30 min at room temperature. After washing with PBS, color development was performed in diaminobenzidine solution (10 mM in 0.05 M Tris-HCl, pH 7.5) with 0.003% H_2O_2.

For the cell line samples, the cells were cytocentrifuged on slide glasses using Cytospin III (Shandon Scientific Ltd., Pittsburgh, PA, USA). After fixation with acetone, immunohistochemical staining was performed as described above. In addition, other fixatives, including paraformaldehyde and Zamboni's fixative, were also tested.

The formalin-fixed, paraffin-embedded tissue specimens were initially deparaffinized and then treated using the heat-induced epitope retrieval method in 10 mM of citrate buffer, pH 6.0; immunohistochemical staining was performed using the CSA system (DAKO) according to the manufacturer's protocol.

Results

Immunohistochemical Staining of CD179a/b in Acetone-fixed Cytocentrifuged Cell Lines

As reported previously and presented in Figure 1, the mAbs HSL96, HSL11, and HSL2 recognized CD179a/VpreB, CD179b/λ5, and conformational pre-BCR, respectively, in membrane-permeabilized cells when analyzed using flow cytometry.[10] We first examined whether these mAbs could also be used for immunohistochemical staining in acetone-fixed cells. When acetone-fixed, cytocentrifuged pre-B-ALL HPB-NULL cells expressing conformational pre-BCR were tested, the HSL11 mAb was able to detect CD179b at a concentration of 5 μg/ml; neither the HSL96 nor the HSL2 mAbs detected this molecule (Figure 1). Typically, a cytoplasmic staining pattern was observed in HPB-NULL cells using HSL11. In contrast, HSL11 did not react with similarly treated Ramos Burkitt cells, which express the complete form of Ig ($\mu\lambda$), but lack the surrogate LCs, suggesting that CD179b binds specifically to SL11.

We also examined the staining patterns produced by commercially available anti-CD179a mAbs: VpreB8, VpreB9, 4G7, and B-MAD-688. When these four anti-CD179a mAbs were examined, only the VpreB8 mAb reacted with CD179a in acetone-fixed HPB-NULL cells (data not shown). However, VpreB8 mAb exhibited a weak nonspecific binding with the nuclei of acetone-fixed Ramos cells at high mAb concentrations. A concentration of 1.25 μg/ml was optimized as a sufficient and specific condition for CD179a detection in precursor B-ALL cells, which does not produce a nonspecific reaction in Burkitt cells (data not shown).

We further examined whether SL11 and VpreB8 could be used for immunohistochemical staining in cells treated with other fixatives and observed that both mAbs react with Zamboni's fixative-treated cells, but not with paraformaldehyde-treated cells (data not shown).

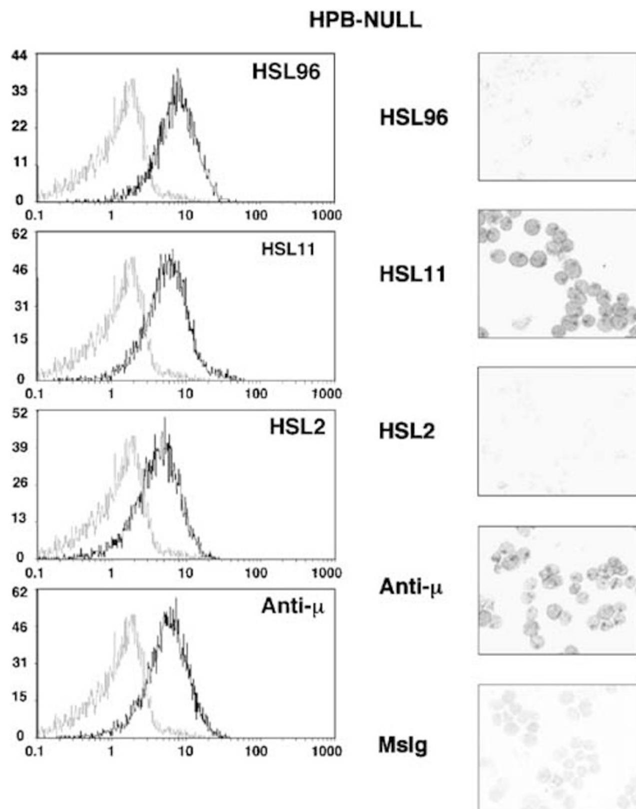

Figure 1 Immunohistochemical detection of CD179b by HSL11 in acetone-fixed, cytocentrifuged precursor B-ALL cell lines. Pre-BCR-expressing HPB-NULL cells were permeabilized and stained with specific mAbs, as indicated, and analyzed using flow cytometry (left panels). The resulting histograms (solid lines) were superimposed on those of the negative control (cells stained with isotype-matched control mouse Ig, broken light lines) and displayed. *X*-axis, fluorescence intensity; *Y*-axis, relative cell number. In parallel, HPB-NULL cells were cytocentrifuged, acetone-fixed, and stained with each mAb, as indicated, using immunohistostaining (right panels). HSL11 is strongly positive and anti-μ is moderately positive, but others are negative. MsIg, iso-type matched control mouse immunoglobulin.

Immunohistochemical Staining of CD179a/b in Acetone-fixed Fresh Frozen Tissues

Next, we used immunohistochemistry to examine whether VpreB8 and HSL11 could detect CD179a/b in clinical childhood B-LBL specimens. When acetone-fixed fresh frozen sections prepared from biopsy specimens obtained from B-LBL patients were examined using immunohistochemical staining, both VpreB8 and HSL11 were found to react with the tissues (Figure 2 and Table 1). Typically, a diffuse cytoplasmic staining pattern was observed in B-LBL tissues using both mAbs (Figure 2). Cases were considered as positive if most of the blasts present in the tissue were clearly stained. As summarized in Table 1, nine out of 10 (90%) B-LBL patients and eight out of 11 (73%) B-LBL patients were positive for VpreB8 and HSL11, respectively. In contrast, no positive staining for VpreB8 or HSL11 was seen in either the Burkitt lymphoma tissues (seven cases) or the diffuse large B-cell lymphoma tissues (three cases), suggesting that both VpreB8 and HSL11 react specifically with B-LBL cells, but not with mature B-cell lymphomas in childhood.

We also examined the other pediatric tumors that must also be included in a differential diagnosis of B-LBL. As presented in Table 2, when acetone-fixed fresh frozen sections prepared from biopsy specimens obtained from seven T-LBL cases, three extramedullary myeloid tumors, and two Ewing sarcoma cases were examined similarly, all of these cases were negative for both VpreB8 and HSL11, indicating the specificity of these mAbs to B-LBL cells.

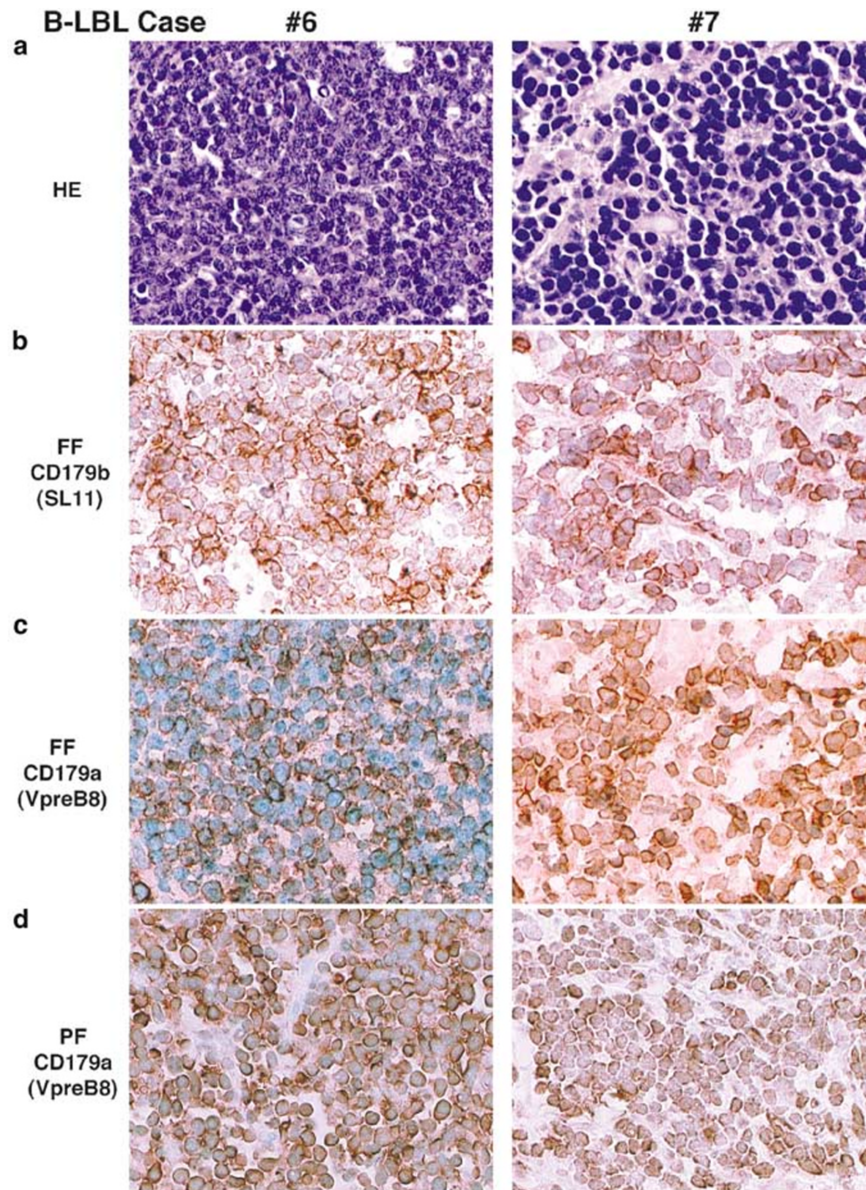

Figure 2 Immunohistochemical detection of CD179a and CD179b. CD179a and CD179b were detected in B-lymphoblastic lymphoma tissues using immunohistochemical staining on acetone-fixed fresh frozen sections ((**b**), (**c**), FF) and formalin-fixed, paraffin-embedded tissue sections ((**d**), PF) from biopsy tissues. The H&E-staining of formalin-fixed and paraffin-embdded tissues is also shown ((**a**), HE).

Immunohistochemical Staining of CD179a in Formalin-fixed, Paraffin-embedded Tissues

Next, we examined whether mAbs against CD179a and CD179b could be used in formalin-fixed, paraffin-embedded tissues. When paraffin-embedded tissues prepared from clinical specimens obtained from B-LBL patients were examined using immunohistochemical staining with the heat-induced epitope retrieval treatment, only VpreB8 reacted with the tissue. The staining results were consistent with those obtained from the immunostaining of acetone-fixed frozen sections. None of the other mAbs reacted with the B-LBL samples. Since higher concentrations of VpreB8 resulted in nonspecific nuclear staining in paraffin sections of Burkitt lymphomas, care must be taken when deciding the appropriate conditions for the use of this mAb.

Discussion

In the current study, we clearly presented that both VpreB8 and HSL11 are useful for the immunohistochemical detection of CD179a and CD179b, respectively, in acetone-fixed B-LBL tissues. Furthermore, VpreB8 can also be used in paraffin-embedded sections. The reactivities of these Abs were highly specific for B-LBL. Reactivity was not seen in tissues of Burkitt lymphoma, diffuse large B-cell

Table 1 Detection of CD179a and CD179b in B-lineage lymphoma tissues using immunohistochemical staining in acetone-fixed fresh frozen sections

Case no.	*Age (years)*	*Sex*	*Origin*	*CD179a*	*CD179b*	*TdT*	*CD34*	*CD19*	*CD79a*	*DR*	*CD20*	μ	*LC*	*CD10*	*CD77*
B-LBL															
1	4	M	Bil-CL	+	+	+	–	+	+	+	+P	–	–	+	–
2[a]	9	M	R-testis	+	+	+	–	+	+	+	+M	NT	NT	+	–
3	7	M	L-CL	+	+	+	–	+	+	+	–	+	–	+	–
4	5	F	L-CL	+	+	+	–	+	+	+	–	–	–	+	–
5	7	F	L-CL	+	+	+	+	+	+	+	–	+	–	+	–
6	1	F	R-CL	+	+	+	–	+	+	+	–	–	–	+	–
7	12	M	AT	+	+	–	–	+	+	+	–	–	–	+	–
8	5	F	L-upper arm	+	–	–	–	+	+	+	+M	–	–	+	NT
9	7	M	L-CL	–	–	+	–	+	+	+	+P	–	–	+	NT
10	4	F	R-radius	+	–	–	+	+	+	+	–	–	–	+	–
11	9	M	CNS	NT	+	+	NT	+	+	NT	NT	+	NT	NT	NT
Burkitt															
1	6	F	AT	–	–	–	–	+	+	+	+	+	–	–	+
2	7	M	AT	–	–	–	–	+	+	+	+	+	Lamda	+	+
3	15	M	AT	–	–	–	–	+	+	+	+	+	Lamda	+	+
4	4	M	AT	–	–	–	–	+	+	+	+	+	Kappa	+	+
5	6	M	AT	–	–	–	–	+	+	+	+	+	Kappa	+	–
6	5	M	AT	–	–	–	–	+	+	+	+	+	Kappa	+	+
7	4	M	AT	–	–	–	–	+	+	+	+	+	Lamda	+	+
B-DL															
1	7	F	R-CL	–	–	–	–	+	+	+	+	+	Lamda	–	–
2	6	M	AT	–	–	–	–	+	+	+	+	+	Lamda	–	–
3	8	M	R-CL	–	–	–	–	+	+	+	+	+	Lamda	+	–

B-LBL, precursor B-cell lymphoblastic lymphoma; DL, diffuse large cell lymphoma; Bil, bilateral; L, left; R, right; CL, cervical lymph nodes; AT, abdominal tumor; LC, light chains; NT, not tested; P, patchy staining pattern; M, membranous staining pattern.
[a]Testicular relapse of precursor B acute lymphoblastic leukemia.

Table 2 Immunohistochemical staining of CD179a and CD179b on acetone-fixed fresh frozen sections of non-B-cell lineage neoplasm tissues

	Positivity	
	CD179a	*CD179b*
T-LBL	0/7	0/7
Extramedullary myeloid tumors		
Granulocytic sarcoma	0/2	0/2
AMoL, skin infiltration	0/1	0/1
Ewing sarcoma	0/2	0/2

T-LBL, precursor T-cell lymphoblastic lymphoma; AMoL, acute monocytic leukemia.

lymphoma, T-LBL, extramedullary myeloid tumors, and Ewing sarcoma.

In pediatrics, the three major types of B-cell lymphoma are B-LBL, Burkitt lymphoma, and diffuse large B-cell lymphoma; the latter two types must be distinguished from B-LBL since the therapeutic protocols for these diseases are quite different from that for B-LBL. In the Berlin Frankfurt Munster (BFM) study group, for example, B-LBL cases were treated using ALL-type protocol with a total therapy duration of at least 24 months.[17] In contrast, mature B-cell lymphoma cases, including Burkitt lymphoma and diffuse large B-cell lymphoma, are treated using a short course of treatment that is usually completed within a year.[18] Each type of B-lineage lymphoma is morphologically unique and distinctive upon histological examination. In the practical pathological diagnosis of lymphomas, however, pathologists may experience difficulties in differentiating B-LBL from other B-lineage lymphomas, especially when only poor-quality biopsy specimens are available.[12] Unfortunately, pathologists are not always familiar with B-LBL because of its rarity among childhood lymphomas; as a result, patients with B-LBL may be misdiagnosed as having mature B-cell lymphoma, such as Burkitt lymphoma. The similarity in marker expression patterns for B-LBL and Burkitt lymphoma is also partly responsible for the risk of misdiagnosis.[11,12]

TdT is considered to be a reliable marker for the diagnosis of cases of precursor lymphocyte origin,[11,12] but TdT is not always positive in B-LBL cases as reported by several different groups.[19–22] For example, Mertelsmann *et al*[20] reported that TdT was absent in approximately 5% of ALL and LBL cases. Orazi *et al*[21] also reported that 6% (two out of 35) of LBL cases was TdT-negative assessed by immunohistochemical staining. On the other hand, CD34 is expressed on human bone marrow progenitor cells and leukemic blasts, and is considered to be an immature marker. Although the expression of CD34 on B-lineage lymphomas suggests their precursor B-cell origin, the positivity of CD34 among the B-LBL cases is approximately 50%. In addition, both TdT

and CD34 are not restricted to the precursor of B cells. In contrast, CD20 is a B-cell-specific marker and its expression increases with B-cell maturation. Therefore, the absence of CD20 expression in B-lineage lymphomas suggests their precursor B-cell origin. However, CD20 expression is variable among cases of B-LBL and approximately 50% of B-LBL cases are CD20-positive, exhibiting sometimes a strong membranous staining pattern.[11] Therefore, it is difficult to specify a B-precursor origin using CD20 expression alone. Based on the above evidences, the development of other markers capable of revealing a precursor B-cell origin is urgently required; in this regard, the results described here are expected to assist in the proper diagnosis of B-LBL among B-cell lymphomas in childhood.

CD179a and CD179b are essential for the development of precursor B cells. Although their biological significance is not fully understood, they are believed to serve as surrogate LCs expressed with μ HCs in pre-BCR to determine whether the clone should survive or die. After subsequent rearrangements in κ or λ LC genes, the expression of the surrogate LCs is suppressed.[6–9] The utilization of such functional molecules in the diagnosis of precursor B-cell lymphomas is appropriate if the expression is conserved even in tumor cells. In precursor B-ALL cells, we previously reported that CD179a, CD179b, and the complete form of pre-BCR were detected by HSL96, HSL11, and HSL2, respectively, and were expressed in most of the CD10-positive precursor B-ALL cases,[10] suggesting that these markers may be useful for the further classification of this disease. Consistent with this observation, CD179a and CD179b, detected by VpreB8 and HSL11, respectively, were frequently expressed in B-LBL cases, whose origin is comparable to that of precursor B-ALL. Thus, the successful employment of these functional molecules in the diagnosis of B-cell lymphomas is another important aspect emphasized in this study.

As shown here, CD179a and CD179b immunohistochemistry can identify more than 90% of B-LBL cases. In our series, the positivity of TdT among the B-LBL cases examined was lower (73%) than that of previous reports.[19–22] The reason for this discrepancy is not known, but it is noteworthy that three TdT-negative cases were positive for either CD179a or CD179b or both. Thus, by combining the TdT and CD179 markers, we believe that virtually all B-LBL cases can be properly judged as having a precursor B-cell origin. The absence of CD179a/b reactivity in Burkitt and diffuse large B-cell-type lymphomas further supports the reliability of this marker.

Occasionally, B-LBL may be misdiagnosed as Ewing sarcoma, since these two diseases have similar morphologies and immunostaining patterns.[23] CD99 (MIC2) was previously considered to be a specific marker for Ewing sarcoma, but this molecule has now been shown to be frequently expressed in B-LBL. Bone tumors with a blastic morphology and a CD45−, CD20−, MIC2+ phenotype can be diagnosed as Ewing sarcoma. In such cases, immunostaining for CD179a/b along with TdT and CD79a will lead to a proper diagnosis. In addition, immunostaining for CD179a/b is also useful for distinguishing B-LBL from either T-LBL or extramedullary myeloid tumors, both of which are included in frequent differential for B-LBL.

Diagnostic markers must be usable in paraffin sections for practical diagnostic procedures. In this regard, the utilization of mAb VpreB8 in paraffin sections, as demonstrated in this report, should facilitate its use in daily diagnostics. Caution must be exercised, however, when using VpreB8 because this antibody may produce nonspecific binding. After careful examination, we selected a concentration of 1.25 μg/ml for our system; however, this value should be evaluated for each laboratory in which the mAb is used, since differences in detection systems may affect the results. Other than VpreB8, unfortunately, none of the mAbs against CD179a/b tested in this study was useful for immunohistochemical detection in paraffin-embedded sections. Since the expression of CD179b was always accompanied by that of CD179a in our cases assessed using fresh frozen section staining (Table 1), paraffin section staining with VpreB8 may be sufficient for the diagnosis of B-LBL. However, the generation of novel mAbs against CD179a/b and preBCR that can react in paraffin sections would be useful and may provide more convincing results.

In conclusion, we have demonstrated that mAbs against CD179a/b specifically detect B-LBL tissues. Although an examination of a larger number of lymphoma tissues is required to confirm their reliability, the application of these mAbs in the immunohistochemical examination of lymphoma tissues should contribute to a precise diagnosis of B-lineage lymphomas.

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