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RESEARCH

A Comprehensive Immunophenotypic Marker Analysis of Hairy Cell Leukemia in Paraffin-Embedded Bone Marrow Trephine Biopsies–A Tissue Microarray Study

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Abstract Hairy cell leukemia (HCL) is an uncommon B cell lymphoproliferation characterized by a unique immunophenotype. Due to low number of circulating neoplastic cells and 'dry tap' aspiration, the diagnosis is often based on BM trephine biopsy. We have performed a consecutive immunohistochemical analysis to evaluate diagnostic usefulness of various HCL markers (CD11c, CD25, CD68, CD103, CD123, CD200, annexin A1, cyclin D1, DBA.44, HBME-1, phospho-ERK1/2, TRAP, and T-bet) currently available against fixation resistant epitopes. We analyzed tissue microarrays consisting of samples gained from 73 small B-cell lymphoma cases, including hairy cell leukemia (HCL) (n=32), HCL variant (HCL-v) (n=4), B-cell chronic lymphocytic leukemia (B-CLL) (n=*11*), lymphoplasmacytic lymphoma (LPL) (n=*3*), mantle cell lymphoma (MCL) (n=*10*), splenic diffuse red pulp small B cell lymphoma (SDRPL) (n=*2*), splenic B cell marginal zone lymphoma (SMZL) (n=*8*), and splenic B cell lymphoma/leukemia, unclassifiable (SBCL) (n=*3*) cases. The HCL cases were 100 % positive for all but 2 (DBA.44 and CD123) of these markers. Annexin A1 showed 100 % specificity and accuracy, which was followed by CD123, pERK, CD103, HBME-1, CD11c, CD25, CD68, cyclin D1, CD200, T-bet, DBA.44, and TRAP, in decreasing order. In conclusion, our results reassured the high specificity of annexin A1 and pERK, as well as the diagnostic value of standard HCL markers of CD11c, CD25, CD103, and CD123 also in paraffin-embedded BM samples. Additional markers, including HBME-1, cyclin D1, CD200, and T-bet also represent valuable tools in the differential diagnosis of HCL and its mimics.

Keywords Hairy cell leukemia · Bone marrow trephine biopsy · Tissue microarray · Immunohistochemistry

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Introduction

Hairy cell leukemia (HCL) is a rare malignant B-cell lymphoproliferation characterized by pancytopenia, splenomegaly, circulating cells with 'hairy' projections, as well as bone marrow (BM) and splenic involvement [1]. By flow cytometry, the neoplastic cells exhibit a unique immunophenotypic profile, showing 'pan B-cell antigen' (e.g. CD19, CD20, and CD22) positivity with consistent co-expression of CD11c, CD25, CD103, and CD123 [1–7]. There are some indolent B cell lymphoma entities, including hairy cell leukemia variant (HCL-v) [8–10], splenic diffuse red pulp small B cell lymphoma (SDRPL) [10, 11], and splenic B cell marginal zone lymphoma (SMZL) [12], which can clinically or morphologically mimic HCL, but, in contrast to it, they are unresponsive to interferon-α- or purine analog-containing treatment protocols [8, 10, 13, 14].

Due to the typically low number of circulating tumor cells, as well as a 'dry tap' at BM aspiration caused by HCL-associated reticulin fibrosis, the diagnosis is often based on the histopathological assessment of BM trephine biopsies [14]. This aspect underscores the clinical importance of carefully elaborated immunophenotypic analysis for HCL also in paraffin-embedded BM samples. There is a broad range of antibodies that are reported to be applicable in fixed and

paraffin-embedded HCL samples, including DBA.44 [15], tartrate-resistant acid phosphatase (TRAP) [16–18], cyclin D1 [19–21], CD11c [22], T-bet [23, 24], and annexin A1 (ANXA1) [25]. Nowadays, a BRAF V600E mutation-specific mouse monoclonal antibody [26], anti-phospho-ERK1/2 (pERK) [27] as well as anti-CD103 [28] rabbit monoclonal antibodies, and, most recently, monoclonal antibody to Hector Battifora mesothelial epitope-1 (HBME-1) [29] have been reported as helpful novel markers for paraffin-embedded HCL samples.

This study was designed to evaluate the diagnostic usefulness of various HCL markers currently available for paraffin sections. We also aimed to define an optimal panel of immunohistochemical stains necessary to reliably differentiate HCL from other villous and non-villous small B-cell lymphomas in BM trephine biopsies.

Materials and Methods

Patients and Tissue Samples

Seventy-three cases, 71 BM trephine biopsy samples and 2 splenectomy specimens, were retrieved from the files of the Laboratory of Tumor Pathology and Molecular Diagnostics, Szeged, Hungary. The cases and specimens studied are summarized in Table 1. The study group comprised HCL (n=32), HCL-v (n=4), SDRPL (n=2), SMZL (n=8), and splenic B-cell lymphoma/leukemia unclassifiable (SBCL) (n=3) cases, moreover BM samples from other small B-cell lymphomas, including B-cell chronic lymphocytic leukemia (B-CLL) (n=11), lymphoplasmacytic lymphoma/Waldenström's macroglobulinemia (LPL/WM) (n=3), and classical mantle cell lymphoma (MCL) (n=10) cases. Each case had been previously diagnosed on the basis of clinical information, histomorphology, flow cytometry, and immunophenotypic characteristics. The diagnosis of 2 SDRPL cases was based on assessment of splenectomy material. The diagnosis of SBCL was used to those splenic small B-cell lymphoma cases where the distinction between SMZL and SDRPL was not possible on the basis of BM evaluation alone.

The BM trephine biopsies were fixed for 12–24 h in neutral buffered formalin supplemented with methanol and glucose (Schaffer's fixative) [30], and decalcified in 12.5 % (w/v) EDTA (Sigma-Aldrich) solution (adjusted to pH 7.0 by cc NaOH) at 60˚C for 16–24 h. The other samples were fixed for 24–72 h in 10 % (v/v) neutral buffered formalin. Each specimen was routinely embedded into paraffin.

Tissue Microarray (TMA) Construction

We constructed TMA blocks with manual Tissue Micro-Array Builder instrument of Histopathology Ltd. (Pécs, Hungary), according to the manufacturer's instructions. Briefly, the recipient paraffin block with 24 holes arranged in four columns and six rows was formed with the TMA Builder. Cores of 2.0 mm from the donor paraffin blocks were punched out with the Paraffin-Punch-Extractor and were arrayed in the recipient paraffin block. Each sample of interest was represented at least in duplicates, to avoid inadequate amount of sampling.

Table 1 Cases and specimens studied

Type of lymphomas	Bone marrow	Spleen	Total
HCL	32	0	32
HCL-v	4	0	4
SDRPL	0	2	2
SMZL	8	0	8
SBCL	3	0	3
MCL	10	0	10
B-CLL	11	0	11
LPL/WM	3	0	3
Total	71	2	73

HCL hairy cell leukemia, *HCL-v* hairy cell leukemia-variant, *SDRPL* splenic diffuse red pulp small B-cell lymphoma, *SMZL* splenic B-cell marginal zone lymphoma, *SBCL* splenic B-cell lymphoma/leukemia, unclassifiable, *MCL* mantle cell lymphoma, *B-CLL* B-cell chronic lymphocytic leukemia, *LPL/WM* lymphoplasmocytic lymphoma/Waldenström's macroglobulinemia

Immunohistochemistry and Evaluation of the Reactions

The immunohistochemical reactions were executed on TMA slides. Briefly, 2 μm-thick paraffin sections were routinely dewaxed, blocked for endogenic peroxidase activities in ethanol containing 1.5 % (v/v) H_2O_2, and heat-treated in appropriate antigen retrieval buffer solutions (Table 2) using a household electronic pressure cooker (Avair IDA). After protein blocking in TRIS-buffered saline (TBS, pH 7.4) containing 5 % (w/v) low fat milk powder, the sections were incubated with the primary antibodies (Table 2) at room temperature for 70 min. Detection was performed using Novolink polymer kit (Leica Biosystems/Novocastra), and nuclear staining was carried out with Mayer's hematoxylin. For primary goat antibodies, a rabbit anti-goat linker antibody (DAKO, Denmark) was used. The immunohistochemical stainings were performed in a 4-channel Freedom Evo liquid handling platform (TECAN, Mannedorf, Switzerland).

In a pilot study, we have tested two distinct monoclonal mouse CD123 antibodies (7G3 and BR4MS), but neither of them has given sufficient sensitivity in HCL cells. To overcome this, a blend of these antibodies (Table 2) was employed afterwards.

The samples were independently assessed by two of the authors (JTL and LK), and in each case with discordant result, consensus was reached by a second look evaluation made

Table 2 List of antibodies used in the study

Antibody/Clone	Source	Origin	Dilution
CD20/polyclonal*	Thermo Scientific	Rabbit	1:1.500
DBA.44**	DAKO	Mouse	1:400
Annexin A1/686106**	R&DSystems	Mouse	1:500
Cyclin D1/polyclonal**	BioGenex	Rabbit	1:200
CD11c/5D11**	Leica/Novocastra	Mouse	1:100
CD25/4C9**	Leica/Novocastra	Mouse	1:500
TRAP/26E5**	Leica/Novocastra	Mouse	1:50
HBME-1*	DAKO	Mouse	1:50
CD103/EPR4166(2) **	Abcam/Epitomics	Rabbit	1:500
Phospho-ERK1/2*	Cell-Signaling	Rabbit	1:500
T-bet/polyclonal**	Santa Cruz	Rabbit	1:1.000
CD123/7G3†**	Becton Dickinson	Mouse	1:100
CD123/BR4MS†**	Leica/Novocastra	Mouse	1:100
CD68/KP1*	Thermo scientific	Mouse	1:100
CD200/polyclonal**	R&DSystems	Goat	1:200

*Antigen retrieval in 10 mM Sodium citrate buffer (0.05 % Tween-20, pH 6.0)

**Antigen retrieval in 10 mM TRIS buffer (0.05 % Tween-20, pH 10.0)

† Admixture of two CD123 antibodies was employed

jointly. On the basis of findings, sensitivity, specificity, and accuracy were calculated (http://en.wikipedia.org/wiki/Sensitivity_and_specificity).

Results

Most of the immunohistochemical markers tested were highly reactive with neoplastic cells of HCL. The antibodies with surface staining highlighted the 'hairy' appearance of the cell membrane. The results with each marker evaluated, including the comparison of antigen expression by immunohistochemical staining in HCL and other small B-cell neoplasms, are summarized in Tables 3 and 4. The specific biological function of tested antigens and their expression pattern in non-neoplastic bone marrow cells are summarized in Table 5.

Anti-CD20 staining was primarily used to assess the scale of BM involvement (Fig. 1a), and tumor cell positivity was detected in all but one cases studied. One rituximab-treated MCL case was found to be CD20-negative.

ANXA1 demonstrated strong cytoplasmic and nuclear staining with 100 % positivity rate in HCL cases with apparently 100 % specificity and accuracy (Fig. 1b), being absent in all non-HCL B-cell neoplasms. On the other hand, it displayed staining with equivalent strength also in granulocytic series, which caused considerable difficulties in the assessment of quite a number of BM samples.

Phospho-ERK antibody was found to be 100 % sensitive for HCL (Fig. 1c), moreover showed 97,6 % specificity and 98.6 % accuracy, having been positive in all HCL and negative in all but one small B-cell lymphomas other than HCL. This marker revealed nuclear and cytoplasmic staining in HCL cells and also in some non-neoplastic mesenchymal cells in the background, which latter feature could be employed as endogenous positive control in the negative cases.

As expected, both CD11c and CD103 antibody revealed 100 % sensitivity in detecting neoplastic cells of HCL. The CD11c/5D11 antibody showed membranous staining with moderate intensity in all but 1 HCL cases (Fig. 1d) as well as weak and mostly focal staining in 1 HCL, 2 HCL-v, 1 SDRPL, and 3 B-CLL cases, making up 85.4 % specificity and 91.8 % accuracy. There was an invariable presence of CD11c-positive non-neoplastic dendritic histiocytes and scattered monocytes in BM and splenic samples, which served as internal control of the staining. The CD103/EPR4166(2) antibody exhibited intense membranous staining in all HCL (Fig. 1e), 3 HCL-v, 1 SDRPL cases as well as weak and focal reactivity in 1 SMZL, providing 87.8 % specificity and 93.2 % accuracy. Apart from the neoplastic HCL cells with membranous positivity, sparse granulocytic cells with weaker membranous and cytoplasmic staining could also be detected using CD103/EPR4166(2).

Immunostaining for CD25 was positive in all HCL cases (Fig. 1f) and negative in all HCL-v, SDRPL, SMZL, and SBCL samples studied. Moreover, 5/11 B-CLL and 5/10 MCL cases were also found to be reactive with the CD25 antibody, leading to 75.6 % specificity and 86.3 % accuracy. Nevertheless, disregarding the B-CLL and MCL cases, the CD25/4C9 antibody had 100 % specificity and accuracy. All positive cases displayed membranous staining with moderate intensity.

All HCL cases showed HBME-1 positivity (Fig. 1g), highlighting the villous surface of the neoplastic cells. In other small B-cell lymphomas, HBME-1 positivity was demonstrated in 2/4 HCL-v, 1/8 SMZL, and 2/2 SDRPL cases, and was found to be negative in all non-splenic small B-cell lymphomas, providing 87.8 % specificity and 93.2 % accuracy. Inhomogeneous or partial staining of neoplastic cells was seen in 7 HCL cases, which can cause difficulties in detecting sparse HCL cells.

Cyclin D1 antibody displayed nuclear staining with variable intensity usually in the majority of the HCL cells (Fig. 1h). Overall, it exhibited 73.2 % specificity and 84.9 % accuracy. However, excluding MCL cases, the cyclin D1 had very high specificity (96.8 %) and accuracy (98.4 %) for HCL.

CD68/KP1, CD200, T-bet, and anti-TRAP antibodies were reactive with HCL cells in all cases, but showed positivity in the majority of non-HCL small B-cell lymphomas too, which findings determined low specificity for these markers. The CD68/KP1 showed granular cytoplasmic staining with

Table 3 Summary of results with immunohistochemical markers studied

	HCL (%)	HCL-v	SMZL	SDRPL	SBCL	B-CLL	LPL/WM	MCL
CD20	32/32 (100)	4/4 (100)	8/8 (100)	2/2 (100)	3/3 (100)	11/11 (100)	3/3 (100)	9/10 (90)
ANXA1	32/32 (100)	0/4 (0)	0/8 (0)	0/2 (0)	0/3 (0)	0/11 (0)	0/3 (0)	0/10 (0)
pERK	32/32 (100)	0/4 (0)	0/8 (0)	0/2 (0)	0/3 (0)	1/11 (9.1)	0/3 (0)	0/10 (0)
CD11c	32/32 (100)	2/4 (25)*	0/8 (0)	1/2 (50)*	0/3 (0)	3/11 (27.3)*	0/3 (0)	0/10 (0)
CD103	32/32 (100)	3/4 (75)	1/8 (12.5)*	1/2 (50)	0/3 (0)	0/11 (0)	0/3 (0)	0/10 (0)
CD25	32/32 (100)	0/4 (0)	0/8 (0)	0/2 (0)	0/3 (0)	5/11 (45.5)	0/3 (0)	5/10 (50)
HBME-1	32/32 (100)	2/4 (50)	1/8 (12.5)*	2/2 (100)	0/3 (0)	0/11 (0)	0/3 (0)	0/10 (0)
Cyclin D1	32/32 (100)	1/4 (25)*	0/8 (0)	0/2 (0)	0/3 (0)	0/11 (0)	0/3 (0)	10/10 (100)
CD68	32/32 (100)	3/4 (75)	2/8 (25)	1/2 (50)	1/3 (33.3)	2/11 (18.2) *	1/3 (33.3) *	0/10 (0)
CD200	32/32 (100)	3/4 (75)	6/8 (75)	1/2 (50)	3/3 (100)	11/11 (100)	3/3 (100)	0/10 (0)
T-bet	32/32 (100)	3/4 (75)	6/8 (75)*	2/2 (100)	2/3 (66.7)*	10/11 (91)*	1/3 (33.3)*	8/10 (80)*
TRAP	32/32 (100)	4/4 (100)	7/8 (87.5)	2/2 (100)	3/3 (100)	10/11 (90.9)	2/3 (66.7)	10/10 (100)
DBA.44	31/32 (96.9)	3/4 (75)	5/8 (62.5)*	2/2 (100)	1/3 (33.3)*	0/11 (0)	0/3 (0)	0/10 (0)
CD123	22/32 (68.8)	0/4 (0)	0/8 (0)	0/2 (0)	0/3 (0)	0/11 (0)	0/3 (0)	0/10 (0)

*Weakly/focally positive

moderate intensity in HCL and other villous lymphoma cases, weak granular staining in B-CLL and LPL cases. It also displayed positive staining in macrophages and myeloid cells which may be disturbing in the evaluation of BM trephines, particularly when the tumor cell burden is low. The CD200 revealed weak to moderate membranous positivity in HCL, all but 4 HCL-v, SMZL, SDRPL, SBCL, B-CLL as well as LPL/WM cases, and was consistently negative in MCL samples. The T-bet antibody demonstrated strong homogenous nuclear positivity in most HCL, HCL-v, and SDRPL, and weaker staining in the remaining positive cases. The anti-TRAP immunostaining gave positive staining in all cases of HCL, and was also identified in almost all (38/41) small B-cell lymphoma cases, having the lowest specificity (7.3 %) and accuracy (47.9 %) in all. The anti-TRAP antibody had weak staining in LPL/WM and B-CLL cases with increasing expression in the proliferation centers and equivalent strong cytoplasmic staining in both HCL and remaining small B-cell lymphoma cases.

All but one (96.9 %) HCL cases were found to be positive with DBA.44, showing predominantly cytoplasmic staining. Of 15 cases of HCL-v, SDRPL, SMZL, and SBCL, 11 were positive (73.3 %), however, all B-CLL, LPL/WM and MCL cases were consistently DBA.44-negative.

The CD123 antibody mixture showed the overall lowest sensitivity, being positive only in 22/32 (68.8 %) of HCL cases, nevertheless its reactivity was 100 % specific and 86.3 % accurate. The positive cells exhibited membranous staining. Apart from HCL cells, scattered monocytes and plasmacytoid dendritic cells were consistently present, serving as endogenous positive control.

Table 4 Specificity and accuracy of the markers studied

	Specificity	Accuracy
ANXA1	100 %	100 %
CD123	100 %	86.3 %
pERK	97.6 %	98.6 %
CD103	87.8 %	93.2 %
HBME-1	87.8 %	93.2 %
CD11c	85.4 %	91.8 %
CD25	75.6 % (100 %*)	86.3 % (100 %*)
CD68	75.6 %	86.3 %
Cyclin D1	73.2 % (96.8 %**)	84.9 % (98.4 %**)
CD200	34.1 %	63 %
T-bet	21.9 %	56.2 %
DBA.44	34.1 %	83.6 %
TRAP	7.3 %	47.9 %
CD20	0	98.6 %

*With exclusion of B-CLL and MCL cases

**With exclusion of MCL cases

Discussion

Diagnosis of HCL is often based on immunomorphological evaluation of BM biopsy specimens. There are indolent B-cell lymphoma cases with dominant BM and splenic involvement, including primary splenic B-cell lymphomas, which can clinically or morphologically mimic HCL, but are unresponsive to treatment options used successfully in HCL [8, 10, 11, 13, 14, 38], therefore detailed immunophenotyping performed in paraffin-embedded BM samples may be of great diagnostic value. In this study, we have performed a consecutive analysis to evaluate the diagnostic usefulness of various HCL markers currently available for paraffin embedded sections including

Table 5 Function and expression pattern of the tested antigens in non-neoplastic bone marrow cells

Antigen	Biological function	Expression in non-neoplastic cells
CD20	Common B-cell antigen	B lymphocytes
DBA.44	B-cell differentiation antigen [15]	Erythroid cells, B lymphocytes
Annexin A1	Phospholipid-binding protein [25]	Granulocytic series
Cyclin D1	Cell cycle regulator [19]	Fibroblasts, endothelial cells
CD11c	Integrin αX chain [22]	Histiocytes, scattered monocytes
CD25	α-chain of interleukin-2 receptor [31]	Activated T lymphocytes
TRAP	Acid phosphatase, isoenzyme 5 [17]	Osteoclasts, macrophages
HBME-1	Unknown [29]	Erythroid precursors
CD103	αE subunit of integrin αEβ7 [32]	Weakly in granulocytic cells
pERK	Phosphorylated kinase protein [33]	Some activated mesenchymal cells
T-bet	Transcription factor [34]	T lymphocytes
CD123	α-chain of interleukin-3 receptor [35]	Plasmacytoid dendritic cells
CD68	Monocyte/macrophage antigen [36]	Monocytes and histiocytes
CD200	Inhibitory protein [37]	Endothelial cells

CD11c, CD25, and CD103 those represent standard constituents of diagnostic HCL panels in flow cytometry [2, 3, 5–7]. To the best of our knowledge, this is the most comprehensive study so far, assessing HCL immunohistochemical markers in paraffin sections.

Upregulation of ANXA1 has been identified through gene expression profiling [39] and, subsequently, it was introduced as a specific immunohistochemical marker of HCL [25]. We found ANXA1 to be apparently 100 % specific, sensitive and accurate, confirming the previous findings however this marker displayed an equivalently strong cytoplasmic and nuclear staining also in the granulocytic series, which might cause considerable difficulties in the assessment of BM samples. Therefore, additional immunohistochemical markers seem to be necessary to apply in the diagnosis of HCL.

Recently, BRAF-V600E mutation has been identified as a disease-defining genetic event in HCL [27]. Subsequently, a novel BRAF V600E mutant-specific antibody has been reported as a highly specific immunohistochemical marker of HCL [26]. Although this antibody is commercially available, due to its rather high price and the availability of a simple, sensitive and inexpensive genetic assay for BRAF V600E mutation [40], we decided not to include antibody VE1 in our study.

The V600E leads to constitutive activation of BRAF that phosphorylates MEK and ERK (the kinase phosphorylated by phospo-MEK) [33]. Immunochemical detection of constant ERK phosphorylation using a pERK-specific rabbit monoclonal antibody has been demonstrated as a sensitive diagnostic assay of HCL [27]. Our results presented here strongly support the diagnostic value of the anti-pERK antibody, being negative in all but one non-HCL cases tested. This marker exhibited nuclear staining in activated non-neoplastic mesenchymal cells, which pattern could be disturbing in evaluating samples with low tumor burden.

CD103 is a cell surface glycoprotein representing αE subunit of the heterodimer integrin αEβ7 [32]. It has been discovered by raising monoclonal antibody B-ly7 directly against HCL [41]. Since then, the anti-CD103 antibodies have been widely used in the diagnosis of HCL by flow cytometry [2, 3, 5–7]. Recently, a novel monoclonal rabbit antibody has been described that can successfully detect neoplastic cells of HCL in formalin-fixed and paraffin-embedded samples [28]. Our study confirmed its diagnostic utility, showing high sensitivity (100 %) in HCL, however, 75 % of HCL-v and about one third of other indolent splenic B-cell lymphoma cases also revealed CD103 positivity. These results coincide with previous findings those have been described by flow cytometric applications [2, 3, 5–7] or in paraffin-embedded samples [28].

Detection of CD11c, the integrin αX chain, has an established diagnostic value in HCL [41, 42]. It is widely used, in combination with CD25 and CD103, for flow cytometry in the diagnosis of HCL [2, 3, 5–7]. Monoclonal antibody 5D11 was introduced for immunohistochemical staining of CD11c antigen in formalin-fixed and paraffin-embedded samples, and has been described as a successively sensitive and specific marker of HCL [22]. In our hands, the CD11c/5D11 antibody showed 100 % sensitivity in HCL samples and also stained 1 case of HCL-v and 1 case of SDRPL with moderate membranous expression in most positive cases. Additionally, 3 of 11 B-CLL cases had weak and focal staining for CD11c in paraffin sections of BM samples, resulted in 85.4 % specificity and 91.8 % accuracy. Our results strongly support applicability of CD11c/5D11 antibody for the diagnosis of HCL in BM trephines fixed in Schaffer's fixative.

Since CD25, the human interleukin-2 receptor alpha chain, has been reported to be positive in HCL [31, 43, 44], it is a widely used flow cytometric marker for the diagnosis of HCL [2, 3, 5–7]. Availability of monoclonal antibody 4C9 allows the qualitative identification of CD25 molecule in paraffin-

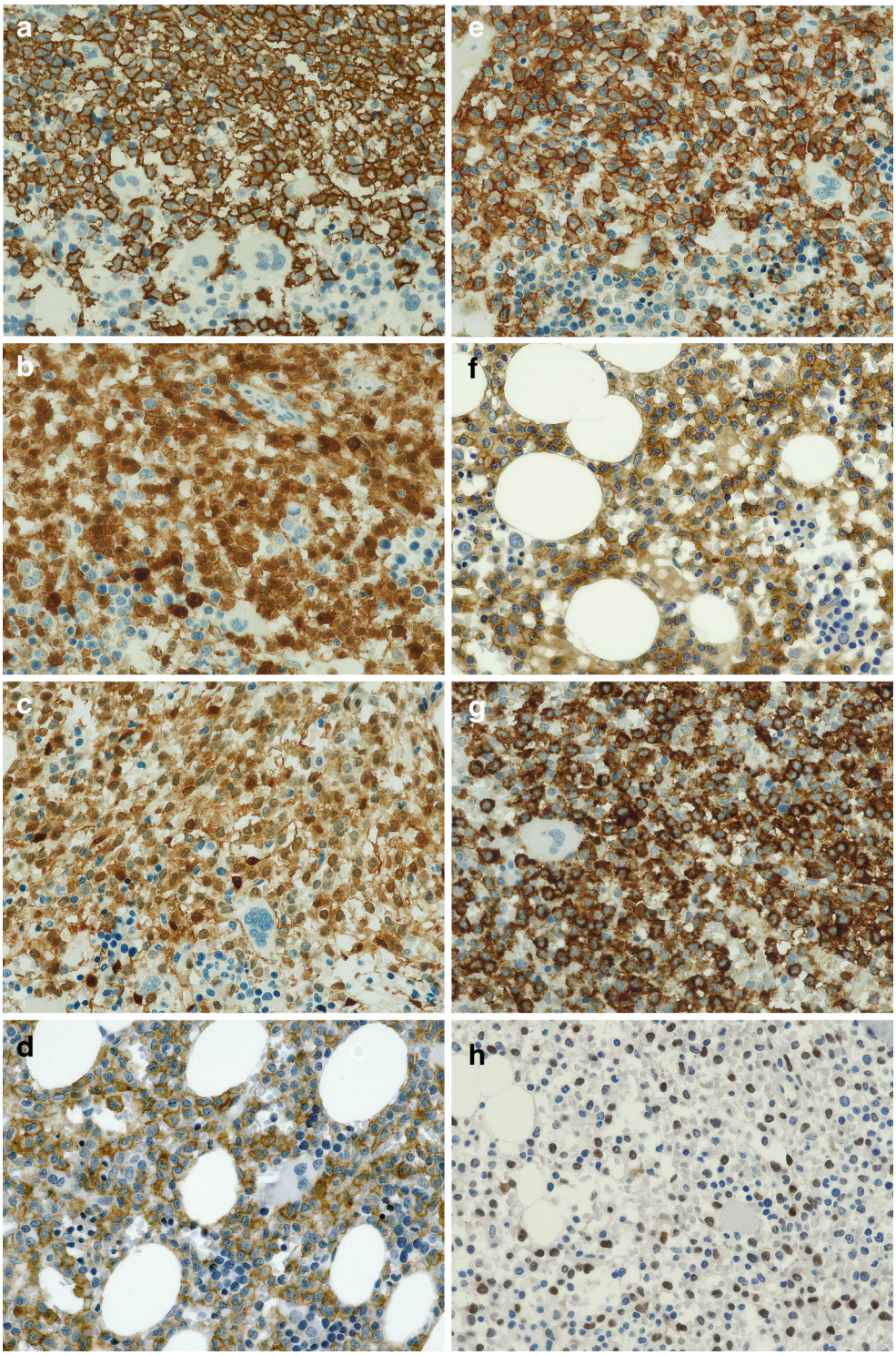

Fig. 1 Characteristic staining patterns of hairy cell leukemia markers studied (×400) (**a**) Strong membranous staining of CD20 bone marrow trephine with HCL infiltration (**b**) Annexin A1 in bone marrow biopsy with hairy cell leukemia. Note the intense nuclear and cytoplasmic staining in both neoplastic and myeloid cells making these two cell population indistinguishable (**c**) Phospho-ERK1/2 positivity occurred as nuclear and cytoplasmic staining in lesional cells (**d**) CD11c membranous positivity in hairy cells (**e**) Strong membranous CD103 staining in bone marrow trephine with marked infiltration by HCL (**f**) CD25 immunostaining showing moderate membranous positivity in HCL cells (**g**) HBME-1 positivity highlighting the villous surface of the neoplastic cells (**h**) Cyclin D1 nuclear staining with variable intensity in HCL cells in bone marrow trephine with marked infiltration

embedded tissue sections, and thus, this antibody is suitable for the diagnosis of HCL in BM trephine biopsies by immunohistochemical staining. Remarkably, to the best of our knowledge, ours is the first study so far using the CD25/4C9 antibody for the evaluation of HCL and its mimics. We found the CD25/4C9 to be a highly sensitive marker for HCL in BM trephine biopsy samples capable of distinguishing HCL and other small B-cell lymphomas, including HCL-v, SMZL, SDRPL, and SBCL. Nevertheless, we observed CD25 positivity also in about half of the CLL and MCL cases, which finding is in accordance with the data reported previously [2, 3, 7, 31, 43]. Apart from neoplastic cells of small B-cell lymphomas, activated non-neoplastic B and T cells also show CD25 positivity which can be troublesome in evaluating BM

samples with low lesional cell content, like in minimal residual disease.

We have recently reported that HBME-1 reacts with a minor subpopulation of B-cells exhibiting villous surface and can be useful in the diagnosis of HCL [29]. Our current findings reinforced its usefulness in HCL as it showed identical results with the CD103/EPR4166(2) antibody. We propose that, HBME-1 represents a low cost immunohistochemical marker of HCL and its mimics, easily accessible in most laboratories. Nevertheless, inhomogeneous or partial staining, found in a proportion of HCL can restrain its applicability when the tumor cell burden is low.

Overexpression of cyclin D1 is a disease-defining event in MCL [19]. The expression of cyclin D1 has been described also in the neoplastic cells of HCL [19–21], but it is not related to overt rearrangements within the BCL-1 locus [45]. In accordance with the previous findings, we demonstrated here that cyclin D1 shows very high specificity and accuracy in HCL too. Since cyclin D1 displays nuclear staining with variable intensity, it might not be suitable to ascertain HCL cells in BM samples with low lesional cell content.

Anti-CD68 antibody KP1 is a widely used monocyte/macrophage marker. Although it is seldom used for this purpose, it has been described to be positive in HCL [36, 46]. In our study, we found the CD68/KP1 to be 100 % sensitive for HCL, but it was positive also in several non-HCL cases, in macrophages, and in myeloid cells which can limit its value in detecting sparse HCL cells.

Monoclonal antibody DBA.44 identifies a fixation-resistant B-cell differentiation antigen, showing positivity in HCL [15]. For years, the DBA.44 was considered to be a standard immunohistochemical stain for the diagnosis of HCL. According to our own observation, this marker cannot differentiate between HCL and its mimics, since most splenic small B-cell lymphoma cases (73.3 %) found to be DBA.44-positive. Moreover scattered, non-neoplastic lymphocytes can be frequently detected with this antibody in reactive conditions, so identifying residual tumor cells with this marker alone is cumbersome [1, 18]. However, all B-CLL, LPL/WM and MCL cases were consistently DBA-44-negative.

Enzyme histochemical detection of TRAP activity has been known as a conventional marker of HCL. Immunohistochemical detection of TRAP has been reported to be highly sensitive in HCL, in particular in combination with DBA.44 [16–18]. In this study, anti-TRAP immunostaining was positive in all cases of HCL and almost all non-HCL small B-cell lymphomas, providing very low diagnostic value in discriminating these neoplasms.

Expression of T-bet, a T-cell-associated transcription factor, has been described in the neoplastic cells of HCL [34] and suggested to be an effective marker of minimal residual disease [23]. In our study, the T-bet exhibited 100 % positivity rate in HCL, but almost all small B-cell lymphomas, both splenic and non-splenic cases, furthermore reactive T cells also demonstrated T-bet positivity, which appears to limit its discriminating value. Nevertheless, its homogeneous strong nuclear staining in HCL is easily recognizable and can be a useful marker in follow-up biopsies, especially in the diagnosis of minimal infiltration by HCL in BM trephines [23].

CD200 (OX-2) was described to be consistently expressed on hairy cells [47]. Yet, its positivity has been widely detected also in other B cell-derived neoplasms [37, 48]. Our study reassured these findings as the CD200 was found to be positive in all HCL and in the majority of other small B-cell lymphoma cases. Our findings confirmed its value in the differential diagnosis of B-CLL and MCL [48] as we showed positivity in all B-CLL and consistent negativity in MCL samples.

Detection of CD123 (α-chain of interleukin-3 receptor) antigen was reported to assist the diagnosis of HCL in flow cytometry [35]. There are monoclonal antibodies available against fixation resistant epitope of CD123 which can facilitate its demonstration in paraffin-embedded HCL samples. In a pilot study, we have tested two distinct monoclonal mouse anti-CD123 antibodies (7G3 and BR4MS), but neither of them has given sufficient sensitivity in HCL cells. Although a blend of these two antibodies resulted in some improvement (not shown), the CD123 still showed the overall lowest sensitivity in our HCL samples. Since, in our hand, both CD123 antibody readily detects plasmacytoid dendritic cells in BM samples, our results suggest that a portion of HCL cases have lower antigenic density which might not be detected by paraffin section immunohistochemistry.

Detection of minimal infiltration, including minimal residual disease of HCL in paraffin-embedded BM biopsy specimens can be of great diagnostic value. ANXA1, being the most specific HCL marker in our series too, displays a strong cross-reactivity with granulocytic series. Phospho-ERK, CD11c, CD25, HBME-1, CD68/KP1, DBA-44, T-bet, CD200, and TRAP exhibited positivity in various non-neoplastic cells which can be troublesome when evaluating low tumor cell content. Inhomogeneous staining with HBME-1, cyclin D1, and CD123 in a proportion of HCL cases can also be a limiting factor. Considering intensity, staining pattern, and distribution, CD103 and T-bet, in combination with CD20, seem to be the most promising markers for follow-up biopsies with minimal infiltration of HCL in BM trephine biopsies, but their usefulness is yet to be delineated.

In conclusion, our results reassured high specificity of ANXA1 and pERK as well as the diagnostic value of standard HCL markers of CD11c, CD25, and CD103 also in paraffin-embedded BM samples. Additional markers, including HBME-1, cyclin D1, CD200, and T-bet also represent valuable tools in the differential diagnosis of HCL. As an optimal panel with high specificity and low cost, we propose the diagnostic application of CD20, ANXA1, pERK, CD103,

and HBME-1 antibodies for paraffin-embedded BM samples suspicious for HCL.

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Conflict of Interest The authors declare that they have no conflict of interest.

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