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ORIGINAL PAPER

Hairy Cell Leukemia-Related Disorders Consistently Show Low CD27 Expression

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Abstract In Japan, typical hairy cell leukemia (HCL) is rare, and HCL-Japanese variant (HCL-JV) is more common. Hairy B-cell lymphoproliferative disorder (HBLD) is another unusual disorder of polyclonal B-lymphocytosis of hairy cell appearance. In the present study, we analyzed the clinical features of 3 patients with HCL, 3 with HCL-JV, and 3 with HBLD. All HBLD patients had the DRB1*04 allele. As compared with other B-cell lymphoproliferative disorders, CD27 expression on B cells was significantly lower in all patients, ranging from 0.3% to 23.4%. Our results suggest that low CD27 expression may be a distinct feature of these HCL-related disorders.

Keywords Hairy cell leukemia · HCL-Japanese variant · HBLD · CD27 · IgVH · DRB1*04

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Introduction

Hairy cell leukemia (HCL) is a distinct B-cell disorder of small lymphoid cells having oval nuclei and abundant cytoplasm with "hairy" projections in bone marrow and peripheral blood [1]. HCL is characterized by pancytopenia, splenomegaly and bone marrow fibrosis, but usually lacks significant lymph node involvement. These B cells usually circulate in low numbers in the peripheral blood and exhibit strong surface expression of CD11c, CD25 and CD103 [1, 2]. HCL-variant (prolymphocytic variant) is a rare B-cell disorder that accounts for 10% of HCL cases in Western countries. Circulating cells have an intermediate morphology between prolymphocytes and hairy cells, and are characterized by a prominent nucleolus [3].

In Japan, typical HCL is rare and the HCL-Japanese variant (HCL-JV) is more common [4]. The HCL-variant in the West and HCL-JV share some features: lymphocytosis without the expression of CD25 and a low response rate to interferon-alpha. Unlike the HCL-prolymphocytic variant, however, cells in HCL-JV have an inconspicuous nucleolus. Machii et al. reviewed 40 Japanese patients with hairy cell disorder: 9 cases were diagnosed as typical HCL, 2 cases as HCL prolymphocytic variant, and the remaining 29 cases as HCL-JV [4].

A unique polyclonal version of B-cell chronic lymphoproliferative disease with hairy cell morphology was first described in 1996 [5]. Machii et al. reported another 4 patients and proposed that HBLD be classified as a new disease entity: hairy B-cell lymphoproliferative disorder (HBLD) [6]. The cell morphology and immunophenotype of HBLD are very similar to those of HCL-JV; however, HBLD patients lack light-chain restriction and clonal immunoglobulin gene rearrangements, indicating

Table 1 Antibody panel

	FITC	PE	PerCP
1	CD5	CD19	CD45
2	CD19	CD10	CD45
3	CD19	CD23	CD45
4	CD19	CD25	CD45
5	CD19	CD27	CD45
6	CD19	CD103	CD45
7	CD19	CD11c	CD45
8	Kappa	Lambda	CD45
9	CD19	IgG	CD45
10	CD19	IgM	CD45
11	CD19	IgD	CD45

FITC indicates fluorescein isothio cyanate, *PE* phycoerythrin, *PerCP* peridinin chlorophyll protein

that the expanding lymphocytes are polyclonal. The majority of HBLD patients have splenomegaly and elevated serum polyclonal IgG. Only 9 patients with HBLD have been reported to date and all have been Japanese [5–9]. Accurate diagnosis of this disorder is crucial to avoid exposing patients to inappropriate treatment.

CD27 is a type I integral membrane glycoprotein composed of a disulfide-linked homodimer of 55-kDa subunits, and is a member of the tumor necrosis factor receptor family. It has been reported that CD27 is a specific marker of memory B cells [10]; CD27 is expressed on most human memory B cells, but not on naive cord blood B cells. In contrast, malignant B lymphocytes occasionally exhibit aberrant CD27 expression [11–14]. In almost all reported typical HCL cases, tumor cells lack CD27 expression in contrast to the positive expression of CD27 in other leukemic B-cell neoplasms [15].

In order to clarify whether reduced CD27 expression is a common feature of both typical HCL and related diseases, we investigated CD27 expression in patients with HCL, HCV-JV, and HBLD, and compared it to that in patients with chronic lymphocytic leukemia (CLL) or low-grade malignant lymphomas in the leukemic phase (leukemic low-grade lymphomas).

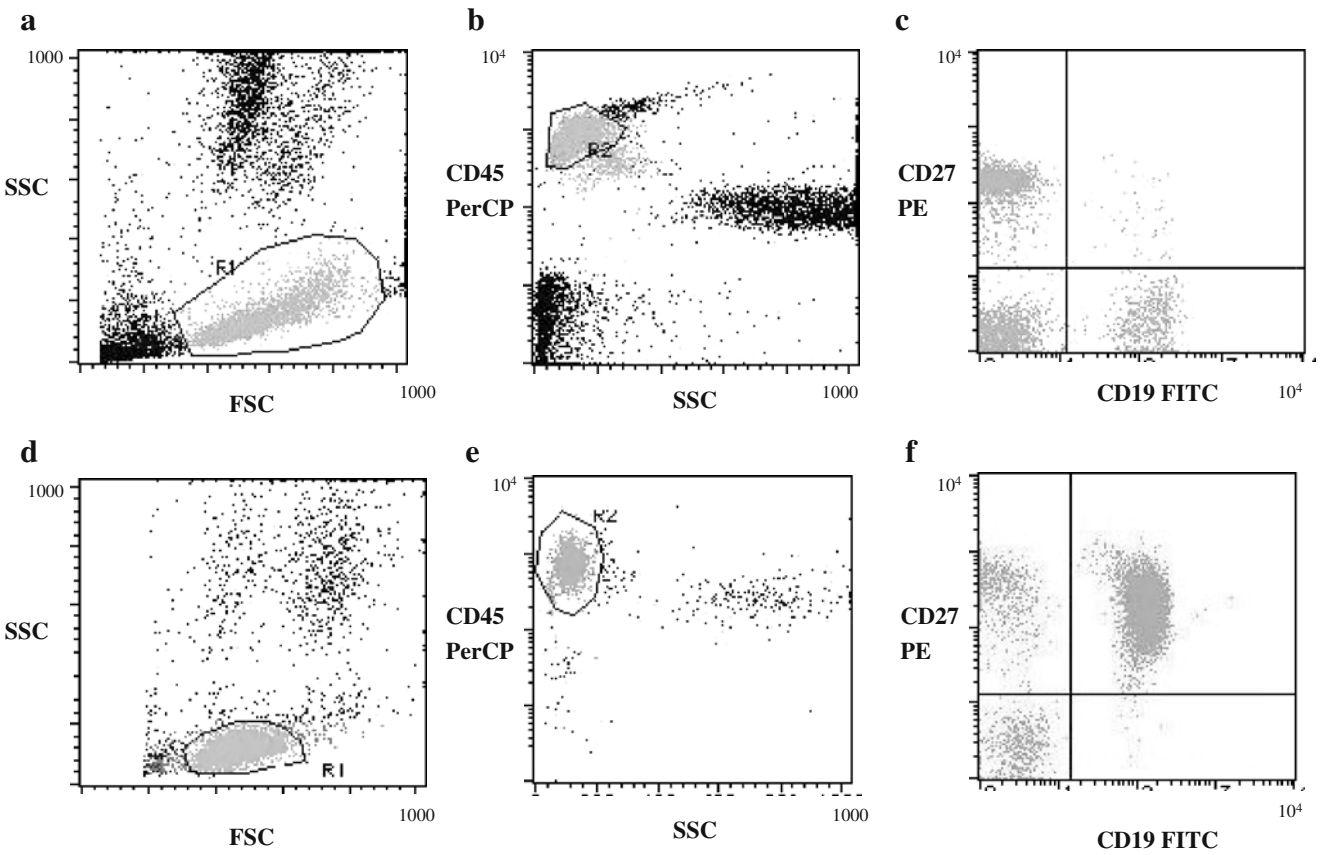

Fig. 1 Flow cytometric analysis of peripheral lymphocyte subsets. Peripheral mononuclear cells in R1 and R2 regions were analyzed for cell-surface expression using the antibody combination described in Table 1. Representative dot plot profiles of low CD27 measured in a patient with HBLD (patient 9 in Table 2; upper **a**–**c** panels) and high CD27 expression in a patient with CLL (lower **d**–**f** panels) are shown. The CD27 and CD19 double positive cells can be identified in the upper right quadrant of the dot plot (**c**, **f**)

Materials and Methods

Patients

The clinical features of 9 patients with HCL, HCL-JV and HBLD are summarized in Table 2. The clinical findings of patient 7 were previously reported as a case report [16]. As control subjects, 30 patients with B-CLL, 12 with MCL, 19 with other low-grade malignant lymphomas in the leukemic phase (7 with lymphoplasmacytic lymphoma (LPL), 7 with splenic marginal zone lymphoma (SMZL), and 5 with follicular lymphoma (FL)) and 10 healthy subjects were included. Diagnosis was based on the WHO classification [1] and all patients had significant peripheral blood B lymphocytes (>20% of lymphocytes as indicated by CD19 positivity). The present study was approved by the Institutional Review Board, and written informed consent was provided by each patient before sampling.

Immunophenotyping

Cell-surface antigens were analyzed by three-color flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), as described previously [17]. In patients with HBLD, mononuclear cells were cultured at 37°C for 8 h before analysis, as described previously [6]. The following fluorescein isothiocyanate (FITC)— or phycoerythrin (PE)-conjugated monoclonal antibodies (BD Pharmingen, San Diego, CA, USA) were used: anti-mouse IgG1 (control), anti-CD5, anti-CD11c, anti-CD19, anti-CD20, anti-CD25, anti-CD27, anti-CD103, anti-IgG, anti-IgM, anti-IgD anti-kappa and anti-lambda. Peridinin chlorophyll (PerCP)-conjugated anti-CD45 antibody (for gating) was purchased from Becton Dickinson.

We analyzed at least 10,000 cells of each patient by the following procedure. A lymphocyte-rich region was set in peripheral mononuclear cells using forward scatter (FSC) and side scatter (SSC), which might include monocytes and debris (R1, Fig. 1a, d). The second lymphocyte-rich region was set around bright CD45-positive and low SSC cells (R2, Fig. 1b, e). Cells in R1 and R2 were analyzed using the antibody sets shown in Table 1.

Detection of Clonal Immunoglobulin VH Gene

Peripheral blood mononuclear cells were prepared using lymphocyte separation medium (IBL, Takasaki, Japan). Collected cells (1×10^7 per tube) were cryopreserved at −70°C until use. DNA was extracted from frozen cells using Sepa Gene (Sanko, Tokyo, Japan) according to the manufacturer's instructions.

In order to evaluate clonality, we examined the variable region of the immunoglobulin heavy chain gene (IgVH) using a polymerase chain reaction (PCR)-based method. PCR was performed using FR1 forward primers and a 6-FAM-labeled JH reverse primer to amplify the sequences between the framework region and joining region of IgVH

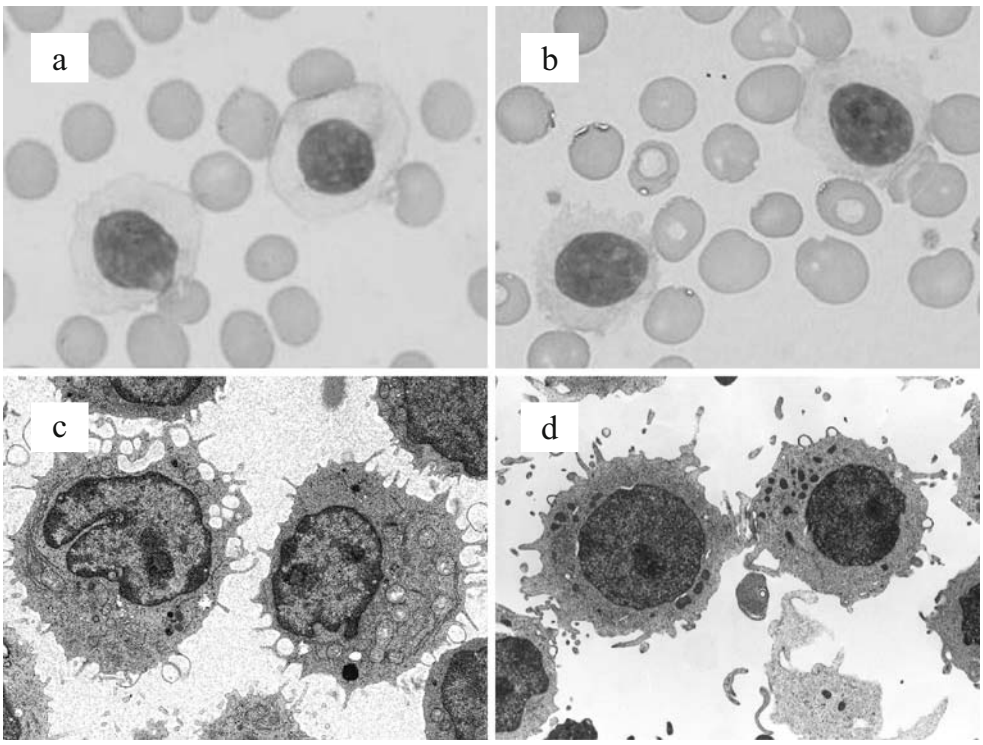

Fig. 2 Abnormal cells in HCL-JV and HBLD patients. Peripheral blood smears from a patient with HCL-JV (**a**: patient 4) and a patient with HBLD (**b**: patient 7) are shown. On electron microscopy, abnormal cells had cytoplasmic projections but no cytoplasmic ribosome lamella complex (**c**: patient 4, **d**: patient 7)

[18]. Amplified products were analyzed using a Genetic Analyzer with GeneScan Software (Applied Biosystems, Foster City, CA, USA).

IgVH Gene Sequencing

Total RNA extraction, cDNA synthesis, and sequencing were performed as described previously [19]. IgVH sequences were classified as non-hypermutated if <2% of sequences deviated from the most common sequence.

HLA Genotyping

High-resolution HLA typing was performed using a reverse sequence-specific oligonucleotide (SSO) DNA typing method with LABType SSO Kits (One Lambda, CA, USA), and a Luminex flow cytometer (Austin, TX, USA).

Statistical Evaluation

CD27 expression in the four groups (HCL and associated disorders, CLL, MCL, and leukemic lymphomas) was compared using one-way factorial analysis of variance (ANOVA). If a significant difference was noted, the Scheffe test was used to determine the significance of differences between each pair of groups. A probability value of $p<0.05$ was considered to indicate statistical significance.

Results

Clinical Features of the Patients

Atypical lymphocytes in the 9 patients with HCL, HCL-JV and HBLD showed abundant cytoplasm with an irregular

Table 2 Clinical features of the patients

Patient number	1	2	3	4	5	6	7	8	9
Diagnosis	HCL	HCL	HCL	HCL-JV	HCL-JV	HCL-JV	HBLD	HBLD	HBLD
Sex	M	M	M	M	M	M	F	F	M
Age	69	63	45	95	80	71	76	77	62
Hb (g/dl)	9.4	9.0	13.9	12.3	14.3	9.9	10.4	9.4	12.4
WBC ($x10^9$/l)	11.5	6.0	12.5	13.9	88.1	50.0	10.9	14.8	11.3
Lymphcyte ($x10^9$/l)	6.9	4.7	11.4	9.7	63.7	47.5	7.9	10.1	6.8
Rate of CD19-positive cells (%)	87.9%	93.1%	98.8%	59.3%	74.7%	64.2%	52.1%	57.6%	21.6%
Plt ($x10^9$/l)	35	113	56	495	107	6.7	127	131	81
Surface antigens (%)*1									
CD11c	94.6	99.7	92.4	ND*2	99.2	99.6	86.7	92.0	95.2
CD25	97.6	93.8	95.6	1.7	1.5	0.2	1.4	2.6	1.1
CD27	6.7	1.3	1.4	17.8	0.3	13.2	23.4	8.9	7.2
CD103	92.7	97.6	15.8	ND	1.4	0.0	0.1	0.1	1.2
Light chain	lambda	double*3	lambda	kappa	null*4	lambda	poly	poly	poly
Heavy chain	IgD	IgM,D,G*5	IgG	IgG	null	IgG	IgG	IgG	IgG
TRAP	(+)	(+)	(+)	(−)	(−)	ND	(−)	(−)	(−)
Serum Ig (mg/dl)									
IgG (870–1700)*6	1670	6620	1610	1910	1100	1071	2620	7240	2200
IgA (110–410)	233	522	125	ND	185	91	261	468	199
IgM (35–220)	56	215	89	110	74	32	849	135	64
Splenomegaly	(+)	(+)	(+)	(−)	(+)	(+)	(−)	(−)	(+)
HLA-DRB1	0405	ND	0803	0901	0405	ND	0407	0405	0405
	0803		0901	1403	1501		1406	1403	1502

*1: mean positive antigen percent in all CD19+cells

*2: not done

*3: simultaneous coexpression of kappa and lambda chain (artifact?)

*4: no detectable light or heavy chain expression

*5: strong IgD, weak IgM and IgG expressions

*6: normal range

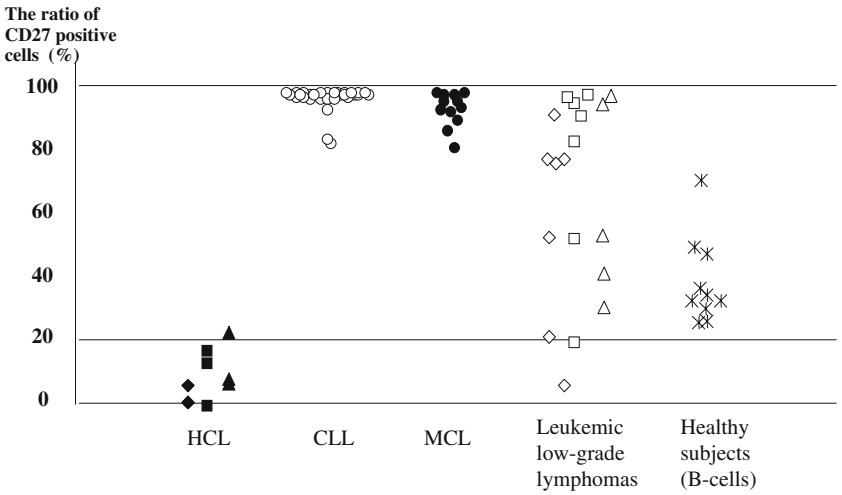

Fig. 3 CD27 expression analysis in HCL, HCL-JV and HBLD. CD27 expression levels on CD19-positive cells in the HCL group were significantly lower than in the CLL group ($p<0.001$*), MCL group ($p<0.001$*), leukemic low-grade lymphoma group ($p<0.001$*) or healthy donor B-cell group ($p<0.05$*). In the HCL group, closed diamonds, closed squares and closed triangles represent HCL, HCL-JV and HBLD, respectively. In the leukemic low-grade lymphoma group, open diamonds, open squares and open triangles represent LPL, SMZL and FL, respectively

edge and an inconspicuous nucleolus on Wright-Giemsa smears of peripheral blood (Fig. 2). Cytoplasmic projections were identified in their cells on phase-contrast microscopy (data not shown) and electron microscopy (Fig. 2). The clinical characteristics of HCL and related disorders are summarized in Table 2. All patients had lymphocytosis with strong CD11c expression and low CD27 expression, as well as the expressions of common B-cell antigens, including CD19 and CD20 (Fig. 1c). Expressions of CD25 and CD103, and tartrate-resistant acid phosphatase reaction (TRAP) were positive only in HCL. Leukemic cells of patients with HCL and HCL-JV expressed single surface kappa or lambda chain, indicating their monoclonal nature, except those in patient 2, in which both chains were expressed, although this was most probably due to artifacts during sample preparation. In contrast, cells from the 3 patients with HBLD lacked a restricted light chain pattern, indicating their polyclonal nature. All patients with HBLD showed increased amounts of serum polyclonal IgG, cell-surface expression of IgG, and HLA-DRB1*04 phenotype (patient 7 had DRB1*0407, and patients 8 and 9 had DRB1*0405).

Table 3 VH gene usage and % homology in HCL and HCL-JV patients

Patient	Diagnosis	VH	% homology
1	HCL	VH3–21	93.0
2	HCL	VH3–30	80.9
3	HCL	VH2–26	96.7
4	HCL-JV	VH3–30	92.2
5	HCL-JV	VH4–59 or 61	89.1
6	HCL-JV	VH5–51	95.0

IgVH Clonality

IgVH clonality was determined by GeneScan analysis and representative fluorescent profiles of patients are obtained. Various peaks with Gaussian-like distribution were noted in patients 7 and 8 (HBLD), indicating polyclonality. Patient 9 (HBLD) was judged to be oligoclonal, as the deviated distribution of peaks with some dominant bands was detected.

CD27 Expression

Surface CD27 expression of CD19-positive cells in the HCL group (HCL, HCL-JV and HBLD) was plotted in comparison with the CLL group, MCL group, or leukemic low-grade lymphoma group (Fig. 3). It is apparent that the expression levels of CD27 were lower in the HCL group, ranging from 0.3% to 23.4%. CD27 expression was particularly high in CLL and MCL, although there was a significant difference in CD27 expression between HCL and leukemic lymphomas. In healthy subjects, the mean value of $CD19^+$ cells in lymphocytes was 9.9% (2.9%–14.6%) and the rate of $CD27^+$ cells in $CD19^+$ cells was 39.5% (25.5%–72.4%).

IgVH Sequence Analysis

Table 3 shows a comparison of the most similar germline sequences, which indicates IgVH gene segment usage and

mutation status. In patients 1–6, the amplified sequences exhibited identical IgVH sequences (indicating that the lymphocytes were truly clonal), and sequence analysis showed that each IgVH gene was hypermutated (range, 80.9% to 96.7%).

Discussion

In the present study, we evaluated the clinical features, cell-surface expression, and IgVH gene status of 3 patients with HCL, 3 with HCL-JV and 3 with HBLD. HBLD is an extremely rare disorder characterized by polyclonal B-cell lymphocytosis with hairy cell appearance. Its clinical features resemble those of HCL-JV; expanded cells express surface IgG and CD11c, but lack CD25 and CD103 expression, and TRAP activity, in contrast to typical HCL [4]. Nine patients with HBLD (7 females and 2 males) have previously been reported in Japan [5–9]. The majority of these 9 patients had lymphocytosis, anemia and/or thrombocytopenia. In the present study, we investigated an additional 3 patients diagnosed with HBLD. Polyclonality was confirmed by cell-surface light chain expression and IgVH gene status (1 patient exhibited deviated IgVH FR1-JH peaks, suggesting oligoclonality). The cell morphology, immunophenotype and clinical characteristics of these 3 patients were typical of HBLD. With regard to the similarities in clinical findings between HCL-JV and HBLD, it is possible that leukemic cells in HCL-JV may be derived from expanded polyclonal lymphocytes in HBLD. Their clinical course should be carefully observed in order to verify this hypothesis.

In 1982, Goldon et al. identified a benign B-cell proliferative disorder in Western countries: persistent polyclonal B-cell lymphocytosis (PPBL) [20]. PPBL is characterized by atypical binucleated B lymphocytes, elevated serum polyclonal IgM, and cell-surface expression of IgM, and patients are predominantly female cigarette smokers. PPBL is closely associated with the HLA-DR7 allele, which was detected in all 3 of the patients reported by Goldon et al. [20] and 24 of the 27 patients reported by Troussard et al. [21] Similarly, in previous studies of HBLD, Matsue et al. reported a patient with the DR4 allele. [5] and Machii et al. found 3 of 4 patients with the DR4 allele [6]. All 3 of the present HBLD patients had the HLA-DR4 allele; patient 7 had DRB1*0407, and patients 8 and 9 had DRB1*0405. These results strongly suggest a close association between DRB1*04 and HBLD, although DRB1*04 is a frequent allele in Japanese, with an incidence of between 27.4% (n=916) [22].

Forconi et al. found that CD27, which is thought to be a specific marker of memory B cells, was low in HCL [15]. By comparing to CLL, MCL and other leukemic lymphomas (Fig. 3), we confirmed the low expression of CD27 in HCL. We also demonstrated for the first time that CD27 expression was low in HCL-JV and HBLD. Thus, low CD27 expression may be a characteristic feature of not only HCL, but also related disorders such as HCL-JV and HBLD. Sequence analysis of the present HCL and HCL-JV patients demonstrated that their IgVH genes were highly somatically mutated. Mutated IgVH has already been reported in the majority of HCL patients [23, 24]. These findings indicate that the memory marker of CD27 expression in leukemic cells is low, despite somatically mutated IgVH genes, which is a definitive genetic marker of memory B cells.

A possible explanation for this discrepancy is that leukemic cells derived from CD27-positive memory cells lose CD27 expression in the process of malignant transformation [11, 12]; for example, neoplastic plasma cells from multiple myeloma patients frequently lose CD27 expression as the disease progresses, whereas plasma cells from healthy subjects strongly express CD27 [13, 14]. Several reports have indicated that leukemic CLL cells show high CD27 expression, irrespective of their IgVH mutation status [25]; however, it is possible that the expanded B cells of HCL and related disorders may originate from unusual normal memory B cells, as the presence of novel CD27-negative memory B cell subsets in healthy individuals has recently been identified [26]. The majority of these memory cells have relatively large cytoplasm, positive IgG surface expression, and mutated IgVH [26].

In conclusion, we studied 9 patients with HCL, HCL-JV and HBLD. All HBLD patients had the DRB1*04 allele, as previously suggested [5, 6]. Surface CD27 expression levels on B cells were significantly lower when compared with CLL, MCL, or other leukemic low-grade B-cell malignancies. Our results suggest that low CD27 expression is a common characteristic among HCL, HCL-JV and HBLD; however, insufficient numbers of patients have been analyzed due to the rarity of the disorders.

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