Frequency of additional clonal populations detected by high sensitivity flow cytometry in patients with hairy cell leukemia

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Abstract Samples from 115 patients with hairy cell leukemia (HCL) were analyzed by high sensitivity flow cytometry for evidence of an additional B cell or plasma cell clone. We found that 10.4% of HCL patients harbored an additional clonal population in either peripheral blood or bone marrow. Among the patients with additional clones, 58% had the second population present at the time of HCL diagnosis and 42% developed the second clone in a subsequent sample. Half of the clonal populations identified represented true disease entities rather than a monoclonal B lymphocytosis (MBL) or monoclonal gammopathy of undetermined significance (MGUS). The frequency of MBL and MGUS was similar to that reported in the general population, but the frequency of B cell-derived malignancies appears increased in the HCL cohort. Compared to patients without additional clones, the patients with second clones were older (median age 66 vs. 55 years old). Patients over age 60 were at higher risk for a second clone (18.2% vs. 5.6%, OR 3.7, $P<0.05$). Among the patients with a second clone, 75% had second B cell clone, 42% had a plasma cell clone, and 17% had both. We have also identified a clear case of bi-clonal HCL; a finding not previously

reported using contemporary diagnostic criteria. We conclude that second clonal expansions are relatively common in patients with HCL, in particular in older patients, and may represent clinically significant neoplasms.

Keywords Hairy cell leukemia · Flow cytometry · B cell lymphoma · Plasma cell neoplasm

Introduction

Hairy cell leukemia (HCL) is a rare blood-, bone marrow-, and spleen-based lymphoproliferative disorder of mature B cells. The neoplastic population can be readily identified immunophenotypically by the expression of CD11c, CD25, and CD103 [1, 2]. Detection of the expression of these antigens on an expanded, clonal B cell population is diagnostic for this disorder. Additionally, the IL3 receptor (CD123) has been proposed as a useful marker for HCL [3]. The origin of the neoplastic cell in hairy cell leukemia is not entirely clear, but the near universal absence of expression of the activation marker CD38, germinal center marker Bcl-6, and germinal center-derived memory cell marker CD27 [4] argue against a germinal center derivation of these cells, despite a not-infrequent expression of CD10 [5, 6].

Patients with HCL are at increased risk for subsequent non-hematopoietic and hematopoietic neoplasms [7–12]. Reasons for the increase are not entirely clear. Immunosuppressive therapy for treatment of HCL, immunosuppressive effect of HCL itself, and/or intrinsic cancer susceptibility of patients with HCL may all play a role.

Aside from the aforementioned epidemiologic studies, reports of second hematologic malignancies in patients with HCL are mostly contained in case reports and series containing very few HCL patients [13–16]. Thus, the true

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incidence of second clonal expansions in HCL is not established. The largest study to date contained 13 HCL and HCL variant (HCL-v) cases from both unselected patient samples and selected patient samples referred to the reference flow cytometry laboratory for confirmation of a second clone. That study demonstrated a second clonal expansion in 4 out of the 13 patients. This finding suggested a possible increased incidence of second clones in HCL, although the study design precluded a definite conclusion [17]. We build on these observations in a much larger study specifically focused on an unselected cohort of HCL patients.

In a retrospective analysis, we have identified 115 individual patients with HCL that had definitive immunophenotypic findings characteristic of HCL (positivity for CD25, CD11c, and CD103 on a clonal mature B cell population) over a 5-year period in a single large academic institution. The frequency of additional clonal hematopoietic proliferations and their immunophenotypic and clinical characteristics are described.

In addition, we detected an unequivocal case of bi-clonal HCL that was confirmed by molecular studies. This, to our knowledge, is the second report of such a case and the first that uses modern diagnostic criteria of HCL. A prior case report of a single patient was published in 1986, and it is not entirely clear whether the patient's diagnosis in that report would meet the modern diagnostic criteria for HCL as it was based on a relatively limited immunophenotyping that would not distinguish HCL from HCL-v [18]. Finally, we took advantage of the large number of cases to further characterize additional immunophenotypic characteristics of HCL.

Materials and methods

Cases selection criteria

This study was approved by the institutional review board at the University of Washington. All immunophenotyping studies were performed at the University of Washington flow cytometry laboratory in the course of routine clinical work. Cases were selected based on the flow cytometric detection of HCL between January 2004 and December 2009. In order to qualify for the study, HCL populations needed to demonstrate monoclonal expression of light chains with bright or moderate co-expression of CD11c, CD25, and CD103. Whenever indicated, clinical and morphologic correlation was performed to confirm the diagnosis. Cases that lacked expression of any of the antigens classically associated with HCL were excluded from further analysis to avoid contamination of the data set with HCL-v and other B cell lymphoproliferative disorders. The cases with a second clone were re-reviewed by two hematopathologists (SC and MR) in order to assure uniform

flow cytometric detection of bi-clonal populations and second B cell or plasma cell clones. Only cases where both reviewers agreed about the unequivocal presence of the second clonal population were included. Clinical and pathologic data for cases with a second clone were then reviewed to determine if the second clone met the 2008 WHO criteria [1] for a true distinct disease entity (B cell lymphoma or plasma cell myeloma) as opposed to monoclonal B lymphocytosis (MBL) or monoclonal gammopathy of undetermined significance (MGUS).

Immunophenotyping

Blood and bone marrow samples were processed using a standard NH_4Cl whole blood lysing technique. Briefly, 100–200 μl of whole blood or bone marrow containing up to 10^6 cells were incubated with 100 μl of a titrated reagent cocktail (see below) and incubated at room temperature in the dark for 15 min. About 1.5 ml of buffered NH_4Cl containing 0.25% ultrapure formaldehyde (Polysciences) was added and incubated in the dark at room temperature for 15 min followed by a single wash with phosphate-buffered saline containing 0.3% bovine serum albumin. Cells (100,000–500,000) were analyzed using two to four 8–10 color diagnostic panels followed by a confirmatory five-color HCL panel. Initial diagnostic workup of bone marrow and peripheral blood samples included a B cell tube, and in some cases, T cell and myeloid tubes. Confirmation of the HCL population was performed using the described HCL tube. A plasma cell tube was added if there was an indication of an abnormal plasma cell population in the initial workup or if there was a clinical suspicion of a monoclonal plasma cell disorder. All antibody reagents were obtained from either Beckman Coulter (Fullerton, CA) or Becton Dickinson (BD) (San Jose, CA). Antibody combinations used for flow cytometric detection of abnormal populations are listed in Table 1.

Cell sorting

Cell sorting was used to separate kappa- and lambda-restricted hairy cell populations in the bi-clonal HCL case. Cells were processed as described under “immunophenotyping”. Kappa- and lambda-restricted populations were individually sorted using flow cytometric sorting on a FACS-ARIA instrument from BD. Cell sorting was performed using the same staining and fixation method as for flow cytometric analysis using kappa-FITC, lambda-PE, and CD19-ECD.

Molecular analysis

Molecular analysis on the individually sorted kappa- and lambda-expressing populations as well as unsorted peripheral

Table 1 Antibody combinations

Panel/fluorochrome	Pacific blue	FITC	PE	ECD	PC5	PC7	A594	APC	APC-A700	APC-CY7
B cells	CD45	Kappa	Lambda	CD19		CD20	CD38	CD10		CD5
T cells	CD45	CD2	CD7	CD34	CD8	CD3	CD4	CD56	CD5	
Myeloid 1	HLA-DR	CD15	CD33	CD19	CD117	CD13	CD38	CD34	CD71	CD45
Myeloid 2	HLA-DR	CD64	CD123	CD4	CD14	CD13	CD38	CD34	CD16	CD45
HCL		CD103	CD25	CD19				CD11c		
Plasma cells	DAPI	cKappa	cLambda	CD19	CD45		CD38	CD56		

blood from the patient with bi-clonal HCL was performed as described below. DNA was prepared using the Puregene kit (Gentra Systems) according to the method of the manufacturer. The DNA segment of interest was amplified by PCR using Biomed-2 framework one to three fluorescently labeled primer sets [19]. The products were then size-fractionated by capillary electrophoresis using Applied Biosystems 3130 Sequence Analyzer. Negative and positive controls were included in each assay.

Results

Characteristics of the study group and HCL immunophenotype

We identified 115 patient samples with a first time flow cytometric diagnosis of HCL at the University of Washington Hematopathology Flow Cytometry Laboratory. Some patients had been diagnosed with HCL prior to presentation at our institution. HCL diagnosis was based on the identification of a clonal B cell population with bright or moderate expression of CD11c, CD25, and CD103. Overall patient characteristics are summarized in Table 2. As expected from previously published reports [20, 21], the disease demonstrated a marked male predominance (M/F ratio >4) and affected mostly middle age to older individuals (median age 56). Approximately 20% of HCL cases demonstrated

aberrant expression of CD10 on at least 10% of abnormal population (expression ranged from uniform to variable). All 29 cases in which CD123 expression was analyzed demonstrated uniform expression of CD123 on the abnormal HCL population.

Immunophenotypic and clinical characterization of second clonal expansions

Twelve of the 115 cases showed flow cytometric evidence of a second clonal population. Compared to patients with HCL alone, the group with a second clone was significantly older [median age 66 (47–85) vs. 55 (27–95), $p<0.05$]. Overall, patients 60 years and older demonstrated a significantly increased risk for a second clone [18.2% vs. 5.6%, odds ratio 3.7 (1.04–13.2), $p<0.05$]. No significant differences were found in sex or immunophenotype (specifically the likelihood of expression of CD10 or CD123) when comparing HCL cases with and without a second clonal population. These findings are summarized in Table 2.

The second clone detected by flow cytometry in this study represented either a second B cell clone or a plasma cell population. The majority of patients with a second clone (8 of 12) had a second clonal B cell population while the remainder had evidence of a plasma cell clone. Of note, two patients had a second B cell clone and second plasma cell clone detected. Notably, half of the second clonal expansions identified represented a true disease entity rather than an MBL or MGUS. A summary of the flow cytometric data is presented in Table 3.

Two patients had multiple myeloma. In patient 1, myeloma was detected at the time of HCL diagnosis with morphology showing greater than 30% plasma cells in the marrow, and in patient 8, plasma cell leukemia was diagnosed post-HCL therapy with greater than 20% plasma cells noted in the peripheral blood. Patient 10 developed chronic lymphocytic leukemia (CLL) and patient 9 developed mantle cell lymphoma. Patient 4 had an abnormal population with a CLL-like immunophenotype representing 18.5% of the white blood cells in the marrow and had a concurrent FISH studies demonstrating trisomy 12. Although this

Table 2 Demographic and immunophenotypic characteristics of patients with HCL

	Without additional clones	With additional clones
Male	82	11
Female	21	1
M/F ratio	4.3:1	11:1
Mean age	55 (27–95)	66 (47–85)
%CD10	21.4 (22/103)	8.3 (1/12)
%CD123	100 (27/27)	100 (2/2)

Table 3 Immunophenotype and clinical characterization of additional clones in HCL patients

	Age	HCL light chain/ clone size by FC	Additional B cell clone	Site	Immunophenotype of additional population/% of WBC
Second clone detected at presentation					
1	47/M	Kappa/4.9%	MM	BM	CD19 (absent), CD45 (absent), CD56 (subset), and cytoplasmic kappa light chain restriction with normal expression of CD38/3.0%
2	52/M	Kappa/1.3%	MGUS	BM	CD19 (absent), monoclonal kappa cytoplasmic light chain restriction with variable CD45, low CD20, and absent CD56/0.3%
3	79/F	Lambda/3.3%	MZL/LPL, MBL, possible MGUS	BM	(1) CD25 and kappa light chain restriction with normal expression of CD45, CD19, and CD20 without CD5, CD10, CD11c, CD103, or FMC-7 (MZL/LPL)/10%; (2) CD5, CD20 (low), CD23, and absent to low level lambda light chain expression with normal expression of CD45 and CD19 without FMC7 or CD10 (MBL) /0.3%; (3) CD19 (variable, subset absent), CD45 (subset decreased), CD56 (subset), and monoclonal kappa cytoplasmic light chain restriction with normal expression of CD38 and CD138 (MGUS)/0.65%
4	75/M	Lambda/11.7%	MBL	BM	CD45, CD19, and CD20, co-expression of CD5, CD38, low FMC7, and low CD25 with dim kappa light chain restriction without CD10, CD103, or CD23 (FISH demonstrated trisomy 12)/18.5%
5	57/M	Kappa/28.6%	MBL	BM	CD5, CD20 (low), CD23 (low to absent), and kappa surface light chain restriction (low) and normal expression of CD45 and CD19 without FMC7/0.7%
6	67/M	Lambda/15.3%	MBL	BM	CD5, CD20, and absent to low kappa surface light chain restriction with normal expression of CD45 and CD19 without significant CD10 or CD38/5.3%
7	76/M	Kappa/76.0%	MBL	PB	CD5, CD20 (low), CD23, and bi-clonal surface light chain restriction (low kappa predominant) and normal expression of CD45 and CD19 without FMC7 or CD10/0.5%
Second clone detected at follow-up					
8	71/M	Lambda/0.005%	MM/plasma cell leukemia	PB	CD19 (absent), CD38 (low), CD45 (variable low), CD56, and monoclonal kappa cytoplasmic light chain restriction/20%
9	85/M	Kappa/0.3%	Mantle cell lymphoma	BM	CD45, CD5, CD19, CD20, low CD38, and lambda light chain restriction without CD10 or CD23 (FISH demonstrated a CCND1 translocation)/25.3%
10	68/M	Lambda/0.2%	CLL	PB	CD5, CD20 (low), CD23, and kappa surface light chain restriction (low) and normal expression of CD45 and CD19 without FMC7 or CD10/60%
11	62/M	Lambda/10.5%	Initially thought to represent a plasma cell neoplasm not further classified; subsequent LN biopsy showed DLBCL with plasmacytic differentiation	BM	Abnormal monoclonal kappa cytoplasmic light chain restriction with normal expression of CD19, CD45, and CD38 without CD56/5.4%. This was initially thought to represent a PCN; however, a subsequent lymph node biopsy demonstrated both an abnormal B cell population with CD20 (variably decreased), CD38 (variably increased), and kappa light chain restriction with normal expression of CD45 and CD19 without CD5 or CD10/6.2% and an abnormal plasma cell population expressing CD45 (increased) and cytoplasmic kappa light chain restriction with normal expression of CD19 and CD38 without CD56/16.7%. The findings in the lymph node were felt to represent DLBCL with plasmacytic differentiation
12	57/M	Kappa/0.005%	MBL	BM	CD5, CD20 (low), CD23, FMC7, and absent surface light chain expression with normal expression of CD45 and CD19 without CD10/0.02%

Abbreviations: *FC* flow cytometry; *HCL* hairy cell leukemia; *PB* peripheral blood; *BM* bone marrow; *MM* multiple myeloma; *MGUS* monoclonal gammopathy of undetermined significance; *LN* lymph node; *MZL* marginal zone lymphoma; *MBL* monoclonal B cell lymphocytosis; *LPL* lymphoplasmacytic lymphoma; *CLL* chronic lymphocytic leukemia; *DLBCL* diffuse large B cell lymphoma

patient was considered to have CLL at the time of the initial flow cytometric study, this patient had a white blood cell

count of 3.9 thousand cells/ μ l with no lymphadenopathy and would therefore not meet WHO 2008 criteria for a

diagnosis of CLL and is classified as an MBL. Patient 3 had three additional clonal populations that included a clonal B cell population negative for CD5, CD10, CD11c, or CD103, a small clonal plasma cell population co-expressing CD56 without CD19, and a small MBL population with a CLL-like immunophenotype. The first population in this patient comprised 10% of the white blood cells by flow cytometry and this patient had an IgM kappa monoclonal spike of 1.2 g/dl on serum protein electrophoresis with immunofixation, findings consistent with a low-grade B cell lymphoma with the immunophenotype and laboratory data suggesting lymphoplasmacytic lymphoma or marginal zone lymphoma (LPL/MZL). The significance of the plasma cell population in this patient is unclear. Although the plasma cell population shared similar light chain expression with the LPL/MZL population, raising the possibility that it represented a component of the B cell neoplasm with plasmacytoid differentiation, a major subset of the plasma cells expressed CD56 without CD19, a phenotype more characteristic of a primary plasma cell neoplasm. The abnormal plasma cell population in this case is therefore classified as possible MGUS. Patient 11 had an abnormal plasma cell population identified in the marrow and was subsequently determined to have a diffuse large B cell lymphoma with plasmacytoid differentiation identified in a lymph node biopsy. In addition, four patients had MBL with CLL-like immunophenotype only and one patient had MGUS only. Most (seven) of the cases demonstrated flow cytometric evidence of the second clone at presentation, while the remaining five cases showed a second clone during follow-up (see Table 3 for details). Of note, two of seven (29%) of the second clones identified at presentation were clinically significant while four of five (80%) of the clones detected at follow-up represented true disease entities. Examples of additional clonal populations are shown in Fig. 1. The prevalence of MBL, MGUS, and of a true B cell or plasma cell neoplasm in this cohort was 5.2% (6/115), 1.7% (2/115), and 5.2% (6/115), respectively.

Bi-clonal hairy cell leukemia

While bi-clonal cases of other B cell neoplasms, most notably CLL/SLL, are encountered with some frequency, to our knowledge only a single case of bi-clonal HCL was reported in the literature over 20 years ago [18]. We have identified an additional case that demonstrated clear bi-clonality based on both alternate light chain expression by two separate HCL populations as well as different immunoglobulin heavy chain receptor gene rearrangements as assessed by polymerase chain reaction. The patient was a 34-year-old male who presented for bone marrow evaluation for investigation of pancytopenia and fever. Flow cytometry demonstrated two immunophenotypically distinct abnormal B cell populations, each with an immunophenotype diagnostic for

Fig. 1 Examples of CLL and MM in combination with HCL, flow dot plots. Additional clonal expansions of either B cells (a) or plasma cells (b) were frequently seen in patients with HCL. An example of CLL in combination with HCL (patient 10 from table 3) is shown in (a). In addition to HCL population (red) showing increased side scatter with abnormal expression of CD45 (increased), CD11c, CD25 and CD103 with lambda light chain restriction and expression of FMC-7, there is also an expanded CLL (magenta) population showing abnormal expression of CD5 and CD23 without FMC-7, significant CD25 or CD103. The CLL clone demonstrated low-level kappa light chain restriction. An example of plasma cell neoplasm in combination with HCL (patient 1 from table 3) is shown in (b). In addition to a HCL population (blue) that showed increased side scatter and kappa light chain restriction with CD25, CD11c and CD103 expression (not shown), there was an abnormal plasma cell population (magenta) with abnormal expression of CD45 (absent), CD19 (absent) and CD56 (subset positive), with cytoplasmic kappa light chain restriction

HCL. The first abnormal B cell population comprised 51.7% of the white blood cells and expressed CD11c, CD19, CD20, CD25, CD103 (variable), and CD123 with kappa light chain restriction without significant CD5, CD10, or CD43. The second abnormal B cell population comprised 13.3% of the white blood cells and expressed CD10, CD11c, CD19, CD20, CD25, CD103 (variable), and CD123 with lambda light chain restriction without significant CD5 or CD43. The immunophenotype of the abnormal B cell populations was consistent with a diagnosis of HCL with two clones—one clone expressing CD10 and lambda light chain and one clone expressing kappa light chain without CD10. Morphologic examination of the bone marrow demonstrated extensive effacement of the normal marrow architecture by abnormal B cells with clumped chromatin and abundant pale cytoplasm on H&E stain. Concurrent peripheral blood demonstrated circulating atypical cells demonstrating distinctive cytoplasmic projections characteristic of HCL. Kappa- and lambda-expressing B cells were separated using flow cytometric sorting and analyzed for a clonal immunoglobulin heavy chain gene rearrangement by polymerase chain reaction followed by capillary electrophoresis. Post-sorting samples clearly demonstrated different clonal immunoglobulin heavy chain gene rearrangements in the two populations. These findings are illustrated in Fig. 2.

Discussion

To our knowledge, this investigation is the first large systematic study analyzing data from unselected cases of HCL that explores the frequency of bi-clonal HCL proliferations or the coexistence of a second B cell or plasma cell clone in patients with HCL by flow cytometry. We documented that more than one in ten patients with HCL harbor one or more additional clonal B cell or plasma cell populations. Moreover, nearly one in five older patients with HCL harbors an additional detectable clone. The frequency of incidental

Fig. 2 Morphologic examination of the bone marrow demonstrated extensive effacement of the normal marrow architecture by abnormal B cells with clumped chromatin and abundant pale cytoplasm on H&E stain and Wright-Giemsa stains (a). Flow cytometry (b) demonstrated two immunophenotypically distinct abnormal B cell populations with immunophenotypes diagnostic for HCL. The first abnormal B cell population expressed kappa light chain restriction (blue) without significant CD10. The second abnormal B cell population expressed

lambda light chain restriction (red) and CD10. Both populations expressed CD11c, CD25, and CD103. Kappa and lambda expressing B cells were separated using flow cytometric sorting and analyzed for a clonal immunoglobulin heavy chain rearrangement by polymerase chain reaction followed by capillary electrophoresis. Post sorting samples clearly demonstrated distinct clonal immunoglobulin heavy chain rearrangements in each population (c)

second clones representing MBL or MGUS (5.2% and 1.7%, respectively) was similar in this cohort of patients with HCL as compared to that previously reported in the literature for healthy adults [22–26]. However, half of the second clones detected (6 of 12) represented true disease entities corresponding to an overall frequency of 5.2% of patients in this cohort. Although it is difficult to find comparable data for the general population, for non-Hodgkin's lymphoma, the reported lifetime risk is 2.13% and the age-adjusted incidence is 19.8 per 100,000 per year, while for myeloma, the reported lifetime risk is 0.65% and the age-adjusted incidence is 5.7 per 100,000 per year as determined by data from the SEER database (calculations based on cases from 2004 to 2008 from 17 SEER geographic regions, see <http://seer.cancer.gov/statfacts/html/nhl.html#prevalence>, <http://seer.cancer.gov/statfacts/html/mulmy.html#risk>). These data suggest that the frequency of B cell lymphoma and/or myeloma may be higher in patients with HCL than in the general population.

More than half (7 of 12) of second clonal populations were detected at presentation and the remaining five at follow up for HCL. From this limited sample, it appears that HCL itself or a predisposition to developing a neoplasm of B cell or plasma cell origin, rather than HCL treatment, may be a primary factor in the development of second clone. This conclusion is supported by prior studies demonstrating lack of association of the presence of a second neoplasm in HCL and a specific therapeutic regimen [7, 12]. However, it is interesting to note that while the majority of second clones detected at presentation were clinically insignificant, the majority detected at follow-up were clinically significant. Although it is tempting to speculate that HCL or HCL treatment may contribute to the progression of indolent clonal expansions to true neoplasms, follow-up longitudinal studies would be needed to test this hypothesis.

The clonal relationship between the HCL clone and additional clone is not entirely clear on the basis of the data available for review. Because this study was retrospective,

we were unable to analyze the populations by molecular methods. The clones were clearly immunophenotypically distinct and there was no significant association between the light chain restriction in HCL and additional clonal population. Whether the two clones shared a more distant precursor is a question that requires further study. In light of recent data suggesting that CLL may represent a stem cell disorder rather than a neoplasm originating in a disordered mature B cell [27] it would be interesting to investigate whether a similar phenomenon may be occurring in HCL.

Our study may not be entirely reflective of a true frequency of second clones in HCL. Observation bias, particularly in patients presenting with symptomatic neoplasms other than HCL and in whom HCL may have been an incidental diagnosis may certainly cause the study to overstate the true prevalence of second clones in HCL. Additionally, as only blood or bone marrow was evaluated in all but one case in this study, we may have missed nodal or extranodal lymphomas. Finally, longer follow-up times may reveal a greater number of cases with a second clone. Nonetheless, our experience is likely to be typical for samples seen in pathology laboratories performing flow cytometric and morphologic evaluation in patients with HCL.

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