

Gene Expression Profiling of Hairy Cell Leukemia Reveals a Phenotype Related to Memory B Cells with Altered Expression of Chemokine and Adhesion Receptors

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Abstract

Hairy cell leukemia (HCL) is a chronic B cell malignancy characterized by the diffuse infiltration of bone marrow and spleen by cells displaying a typical "hairy" morphology. However, the nature of the HCL phenotype and its relationship to normal B cells and to other lymphoma subtypes remains unclear. Using gene expression profiling, we show here that HCL displays a homogeneous pattern of gene expression, which is clearly distinct from that of other B cell non-Hodgkin lymphomas. Comparison with the gene expression profiles of purified normal B cell subpopulations, including germinal center (GC), pre-GC (naive), and post-GC (memory) B cells, shows that HCL cells are more related to memory cells, suggesting a derivation from this B cell population. Notably, when compared with memory cells, HCL cells displayed a remarkable conservation in proliferation, apoptosis, and DNA metabolism programs, whereas they appeared significantly altered in the expression of genes controlling cell adhesion and response to chemokines. Finally, these analyses have identified several genes that are specifically expressed in HCL and whose expression was confirmed at the protein level by immunocytochemical analysis of primary HCL cases. These results have biological implications relevant to the pathogenesis of this malignancy as well as clinical implications for its diagnosis and therapy.

Key words: DNA microarray • germinal center • hairy morphology • marrow fibrosis • homing

Introduction

Hairy cell leukemia (HCL) is a chronic B cell lymphoproliferative disorder characterized by marked splenomegaly, pancytopenia, a low number of circulating tumor cells and diffuse infiltration of the spleen, liver, and BM by tumor cells exhibiting a hairy appearance and a phenotype different from that of other B cell lymphomas (1–4). HCL is highly sensitive to interferon and purine analogue-based treatments (5). Despite the important advances in diagnosis and therapy, the origin, biological characteristics, and pathogenesis of HCL remain obscure.

Phenotypically, HCL cells do not clearly resemble any of the known B cell subpopulation. In the majority of the cases, leukemic hairy cells bear somatic point mutations in their Ig variable genes (6, 7), indicating that the cell giving rise to HCL has transited through the germinal center (GC) of peripheral lymphoid organs, where it has been exposed to the hypermutation mechanism (8). Because HCL does not display significant ongoing somatic hypermutation (6, 7), it has been suggested an origin from a post-GC B cell

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Abbreviations used in this paper: APAAP, alkaline phosphatase antialkaline phosphatase; B-CLL, B cell chronic lymphocytic leukemia; B-NHL, B cell non-Hodgkin lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; Gas, growth arrest-specific; GC, germinal center; HCL, hairy cell leukemia; LCL, lymphoblastic cell line; MCL, mantle cell lymphoma; MM, multiple myeloma.

(4). However, an origin within the GC has also been suggested based on the recent finding that a fraction of cases express multiple and clonally related surface Ig isotypes (6, 7), which is consistent with an arrest at the stage of Ig isotype switch (7), which occurs in the GC. Thus, the cell of origin of HCL is still uncertain.

The uncertainty about the phenotype of HCL is further fueled by several distinctive properties of these cells, including their consistent association with marrow fibrosis, their hairy appearance, their selective homing to BM, liver, and spleen with sparing of lymph nodes, and their high sensitivity to treatment with interferons and purine analogues (1–4). These features suggest that the transformed phenotype of HCL may involve a complex dependence from the microenvironment.

To gain insight into these issues, we investigated the gene expression profiles of 14 cases of HCL using oligonucleotide-based DNA microarrays representative of ~12,000 genes. The gene expression profiles of HCL have been comparatively analyzed with normal and neoplastic B cell populations to: (a) identify the cell of origin of HCL; (b) determine its relationship with other B cell malignancies; (c) dissect the cellular programs that are altered as a consequence of malignant transformation; and (d) identify genes and proteins that are specifically expressed in HCL.

Materials and Methods

Cases. The clinico-pathological features of the 14 HCL cases included in this work are shown in Table SI (available at <http://www.jem.org/cgi/content/full/jem.20031175/DC1>). At the time of sample collection, 10 cases were at diagnosis, whereas the others had been treated previously with different modalities (Table SI), but were off therapy for at least 1 yr (supplemental Materials and Methods contains a detailed description of sample collection and preparation, available at <http://www.jem.org/cgi/content/full/jem.20031175/DC1>).

This analysis also included 10 cases of B cell chronic lymphocytic leukemia (B-CLL) and 36 cases of B cell non-Hodgkin lymphomas (B-NHLs), which were classified as follicular lymphomas (FLs; $n = 6$), mantle cell lymphomas (MCLs; $n = 10$), Burkitt lymphomas (BLs; $n = 4$), and diffuse large B cell lymphomas (DLBCLs; $n = 16$), according to the REAL and WHO classifications (3, 4). Except for MCLs, the detailed characterization of these cases has been reported previously (9). The normal B cell subpopulations have also been described previously (9, 10). Informed consent was obtained from the patients, and the tissue collection was approved by each institutional ethical committee. The analyses include the gene expression profiles of four cell lines derived from multiple myeloma (MM; F24, JJN3, SKMM1, and SKMM2) and five lymphoblastoid cell lines (CB33, RD, Daikiki, IARC304, and NC6).

Generation of Gene Expression Profiles. Total RNA was extracted using the TRIzol reagent (Invitrogen and Life Technologies) and purified using the Rneasy Kit (QIAGEN). Double-strand cDNA was generated from 5 μ g of total RNA using the SuperScript Choice System (Invitrogen and Life Technologies) and a poly-dT oligonucleotide that contains a T7 RNA polymerase initiation site. The double-strand cDNA was used as template to gen-

erate biotinylated cRNA by in vitro transcription using MEGA-script T7 High Yield Transcription kit (Ambion), biotin-11-CTP, and biotin-11-UTP (PerkinElmer). The biotinylated cRNA was purified by the Rneasy Kit (QIAGEN) and fragmented according to the Affymetrix, Inc. protocol. 15 μ g of fragmented cRNA was hybridized to U95Av2 microarrays (Affymetrix, Inc.). The gene expression values were determined by software using the Global Scaling option (Microarray Suite 5.0; Affymetrix, Inc.).

Gene Expression Profiles Analysis. The dendrogram (see Fig. 1) is generated using a hierarchical clustering algorithm based on the average-linkage method (11, 12). Only genes displaying a \geq twofold average change in the expression level across the whole panel were chosen to generate the hierarchical clustering. The expression value of each selected gene is normalized to have a zero mean value and unit standard deviation. The distance between two individual samples is calculated by Pearson distance with the normalized expression values. To perform the supervised gene expression analysis (see Fig. 2 A–D, and Figs. 3 and 4), we used the Genes@Work software platform, which is a gene expression analysis tool based on the pattern discovery algorithm structural pattern localization analysis by sequential histograms (13, 14). The classification method used for the cell type classification (see Fig. 2 E) was described previously (9). In brief, the classifier is a scoring function based on the values of a set of genes (gene cluster), which are differentially expressed in two sets of cell types and, thus, can be used for cell type classification. The higher the score, the more likely it is that a cell type is related to the phenotype set.

Immunohistological Stainings. Immunophenotype was established in all cases at the time of diagnosis (Table SII, available at <http://www.jem.org/cgi/content/full/jem.20031175/DC1>), and in most cases, immunohistochemical studies were directly performed on paraffin sections from BM trephines (supplemental Materials and Methods contains detailed information about the antibodies used).

Immunostaining procedures were performed as follows. BM paraffin sections (3–5 μ m thick) were attached on silane-coated slides, dewaxed in three changes of xylene, dehydrated, and subjected to microwaving (750 W \times three cycles of 5 min each) using 1 mmol/L EDTA buffer, pH 8.0, as antigen retrieval solution (15). After microwave heating, sections were allowed to cool at room temperature for ~20 min, washed with Tris-buffered saline, and stained by the three-stage alkaline phosphatase antialkaline phosphatase (APAAP) technique (for mAb detection) or the four-stage APAAP procedure (for polyclonal antibody detection; references 15, 16). The same staining procedures were used to label acetone-fixed frozen sections from undecalcified BM trephines or cytospin preparations (17, 18). In all instances, slides were counterstained for 5 min in hematoxylin and mounted in Kaiser's glycerol gelatin.

Online Supplemental Material. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20031175/DC1>. The supplemental material provides: (a) description of the clinical and pathological features of the HCL cases (Table S1); (b) immunophenotypical characterization of the cases (Table S2); and (c) details on sample processing, isolation of CD19⁺ B cells from peripheral blood and antibodies used (supplemental Materials and Methods).

Results

Gene expression profile analysis was performed on 16 HCL samples obtained from 14 different patients: 11 sam-

ples were represented by BM biopsies, 3 by CD19⁺ purified B cells from peripheral blood (for 2 samples, the BM biopsy is also depicted), and 2 by mononucleated cells from peripheral blood and spleen, respectively. 6 cases of FL, 4 cases of BL, 16 cases of DLBCL, 10 cases of MCL, and 10 cases of B-CLL were included in the comparative analysis.

HCL Cells Display a Homogeneous Phenotype Distinct from Other B Cell Malignancies. To investigate whether HCL represents a single disease entity and to determine its relationship to other B cell malignancies, we used an unsupervised clustering method (Fig. 1; references 11, 12). Fig. 1 A shows that this method promptly distinguishes HCL from all other B cell malignancies analyzed. Within the HCL region, the samples clustered based on their tissue of origin, namely BM (Fig. 1 A, HCL BM biopsies) or peripheral blood (including the three purified samples) and spleen. The BM-derived samples displayed a prominent set of expressed genes collectively identified as “bone marrow signature” in Fig. 1 A. To exclude the possibility that the separation of the HCL samples from the other malignancies was due to the specific effect of contaminant tissue, the analysis was performed eliminating the HCL BM biopsies. The re-

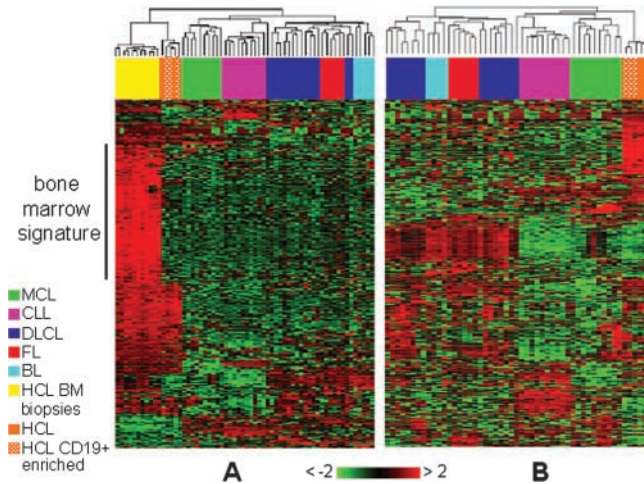


Figure 1. Unsupervised hierarchical clustering of gene expression profiles generated from HCL, non-Hodgkin lymphomas, and B-CLL. Unsupervised analysis was performed on 16 HCL samples obtained from 14 different patients as follows: 11 samples are BM biopsies (HCL BM biopsies) and 5 samples (HCL) are from different origins, including 3 samples of CD19⁺ purified cells from peripheral blood (for two of them, the BM biopsy is also depicted), 1 sample of mononucleated cells from peripheral blood, and 1 sample of spleen biopsy. The representative panel of B cell malignancies includes 6 cases of follicular lymphoma (FL), 4 cases of Burkitt lymphoma (BL), 16 cases of diffuse large B cell lymphoma (DLBCL), 10 cases of mantle cell lymphoma (MCL), and 10 cases of B cell chronic lymphocytic leukemia (B-CLL). The dendrograms are generated using a hierarchical clustering algorithm based on the average-linkage method. In the matrix, each column represents a sample and each row represents a gene. The color scale bar shows the relative gene expression changes normalized by the standard deviation (0 is the mean expression level of a given gene). (A) The 62 tumor samples (16 HCL, 6 FL, 4 BL, 16 DLBCL, 10 MCL, and 10 B-CLL) are clustered according to their expression of 382 genes. (bone marrow signature) Genes specifically associated to the BM biopsies. (B) The HCL BM biopsies are not included in this analysis. The samples are clustered according to their expression of 389 genes.

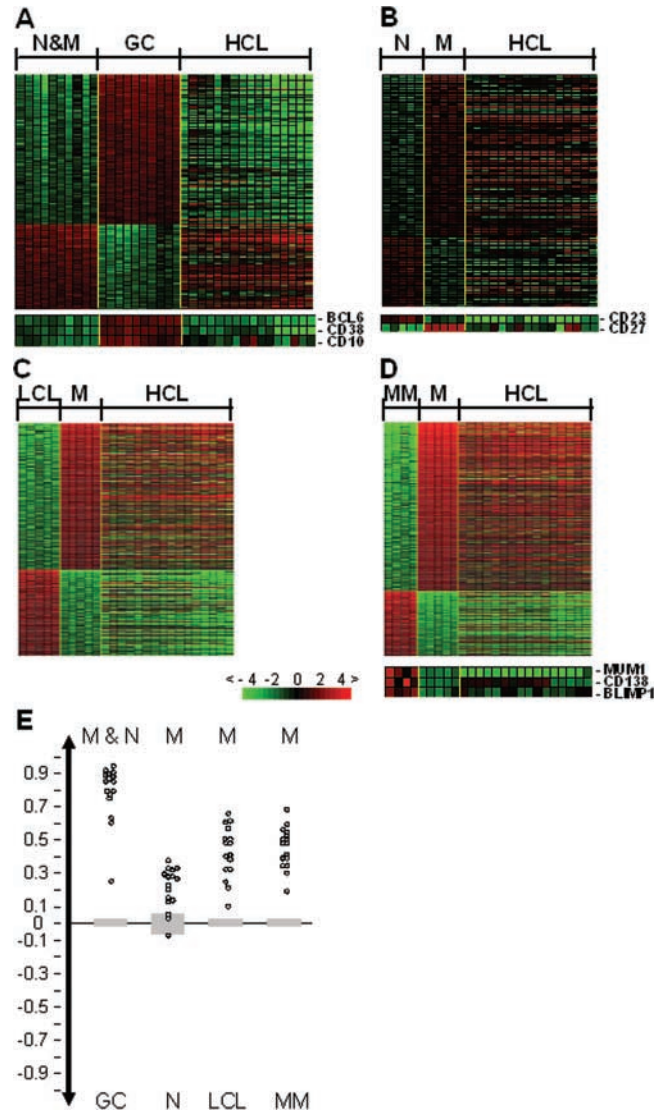


Figure 2. Relatedness of the gene expression profile of HCL to normal B cell populations, and LCLs and multiple myeloma (MM) cell lines. A supervised analysis is used to identify the genes differentially expressed between two groups of samples. (A) Naive and memory B cells (N & M) are compared with GC centroblasts and centrocytes. (B) Naive B cells are compared with memory B cells. (C) EBV-transformed LCLs, representing immunoblasts, are compared with memory B cells. (D) MM cell lines, representing transformed plasma cells, are compared with memory B cells. The expression of the selected genes is investigated in HCL represented on the right side of each matrix (A–D). The expression of specific GC (BCL6, CD38, and CD10), naive (CD23), memory B cell (CD27), and plasma cell (MUM1, CD138, and BLIMP1) markers are highlighted (A–D, bottom). The supervised analysis is performed using the Genes@Work software platform. The results of the analysis are shown in the matrices, where each row represents a gene and each column represents a sample. Genes are ranked according to the z score (mean expression difference of the gene between the phenotype group and the control group/standard deviation). The color change in each row represents the gene expression relative to the mean across the samples. Values are visualized according to the scale bar that represents the difference in the ζ score (expression difference/standard deviation) relative to the mean. (E) A cell-type classification is used to measure the relatedness of HCL to memory and naive B cells (M & N) or GC, to memory (M) or naive (N) B cells, to memory B cells or LCL cell lines, and to memory B cells and MM cell lines. The gray area marks 95% of confidence: the p-value decreases with increasing distance from the x axis.

sults (Fig. 1 B) show that the remaining HCL samples, including the purified samples, still cluster separately from the other B cell malignancies, indicating that the BM signature is not responsible for the separate HCL clustering. The non-HCL B cell malignancies are split into two main branches according to their GC (FL, BL, and DLBCL) or non-GC (MCL and B-CLL) origin (9, 19, 20). Overall, this analysis demonstrates that HCL cases show a largely homogeneous phenotype distinct from other B cell malignancies.

HCL Are Related to Memory B Cells. To investigate the cellular derivation, we analyzed HCL for the expression of genes that are differentially expressed in purified tonsillar GC (centroblasts and centrocytes) versus non-GC B cells (naive and memory), as defined previously by supervised analysis (10). The analysis indicated that the HCL cases are significantly more related to memory and naive B cells than to GC B cells (Fig. 2, A and E). An analogous analysis performed using a profile of genes differentially expressed between naive and memory B cells showed that hairy leukemic cells are more related to memory B cells than to naive B cells (Fig. 2, B and E). To further dissect their post-GC phenotype, we analyzed the HCL profile for genes that are differentially expressed between EBV-transformed lymphoblastic cell lines (LCLs) and MM cell lines as representative of immunoblasts and plasma cells, respectively. The results indicate that HCL is more related to LCLs than to MM cell lines (unpublished data). When the comparison was performed between LCLs or MM cell lines and memory B cells, HCL proved to be more related to memory B cells (Fig. 2, C–E). The expression of specific GC (CD10, BCL-6, and CD38), naive (CD23), memory B cell (CD27), and plasma cell (MUM-1, CD138, and BLIMP-1)

markers (Fig. 2 A, B and D, bottom) confirmed the absence in HCL of GC and plasma cell markers as reported in previous immunohistochemical papers (3, 4, 21, 22). The memory B cell marker CD27 (23) is present in most of the HCL samples, although the expression levels are heterogeneous and lower than in memory B cells. Together, these results indicate that HCL is more related to memory B cells than to other types of known B cell subpopulations. Thus, this malignancy may derive from the transformation and clonal expansion of a memory B cell.

HCL Cells Differ from Memory B Cells in the Expression of Cytokines and Chemokine and Adhesion Receptors. To further investigate the transformed phenotype of HCL vis a vis the expression of genes that characterize normal mature B cells, we examined the difference in the pattern of expression of those genes that are differentially expressed in naive, memory, and GC cells during the GC transit. These genes were classified according to relevant functional or operational categories (10). Consistent with their relatedness (Fig. 2), HCL showed a gene expression profile largely superimposable to that of memory B cells in the proliferation, activation, apoptosis, and DNA metabolism programs (Fig. 3). Within the proliferation program, the exceptions of cyclin D1 and CHC1L are notable, which were clearly up-regulated in HCL. CHC1L, which encodes for a guanine nucleotide exchange factor, and cyclin D1 were also identified among the genes specifically overexpressed in HCL cells (see Fig. 4). HCL cells differed from memory B cells in the expression of various genes encoding signaling molecules and transcription factors.

Notably, the major differences between HCL and memory B cells involved the expression of genes encoding cyto-

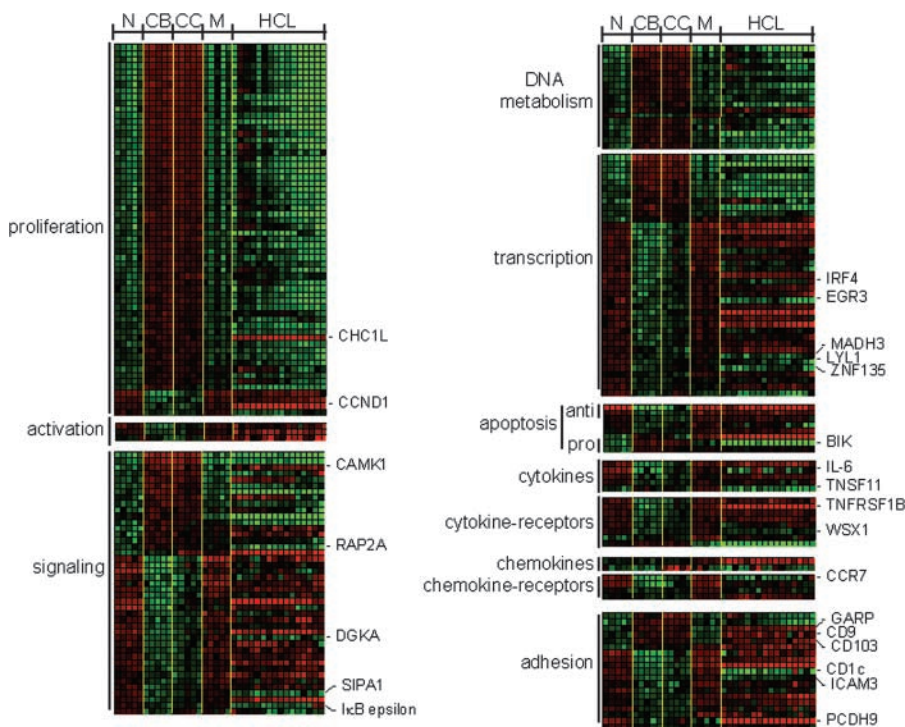


Figure 3. Analysis in HCL of genes associated in normal B cells to the GC transitions. The genes that are differentially expressed in naive, memory, and GC B cells during the GC transit were identified by supervised analysis. The genes were classified according to functional categories. The expression of the transition genes is investigated in HCL (right, both matrices). For description of the matrix, see Fig. 2. Genes that show different expression (at least threefold) between memory B cells and HCL are indicated. For the complete list of the genes, GenBank/EMBL/DBJ accession nos., and Affymetrix codes, see Table S3 (available at <http://www.jem.org/cgi/content/full/jem.20031175/DC1>).

kines and chemokines and their receptors, as well as genes encoding adhesion molecules. In particular, HCL cells do not express the TNSF11 gene (known as RANKL) that is expressed in memory B cells, and it is known to be involved in lymphocyte development and lymph node organogenesis (24). HCL cells also showed relatively low expression of the CCR7 gene, which encodes a chemokine receptor involved in B cell homing (25). Several genes encoding adhesion molecules absent in memory B cells are expressed in HCL cells (GARP, CD9, CD103, and PCDH9), whereas RNA species expressed in memory B cells are not expressed in HCL (CD1c and ICAM3), sug-

gesting the acquisition of peculiar adhesion property by HCL (see Discussion).

Identification of Genes Specifically Expressed in HCL. To identify genes whose expression is specifically associated with HCL, we used a supervised analysis comparing HCL versus various normal B cell subpopulations (naive, GC, and memory) and major subtypes of B cell malignancies (6 FL, 4 BL, 16 DLBCL, 10 MCL, and 10 B-CLL). We used 10 samples of HCL, including 5 BM biopsies and 5 non-BM biopsies (cases 1–4, 6, 8, 11, 12, and the CD19⁺ enriched samples of cases 13 and 14). The analysis identified 89 genes (7 down-regulated and 82 up-regulated)

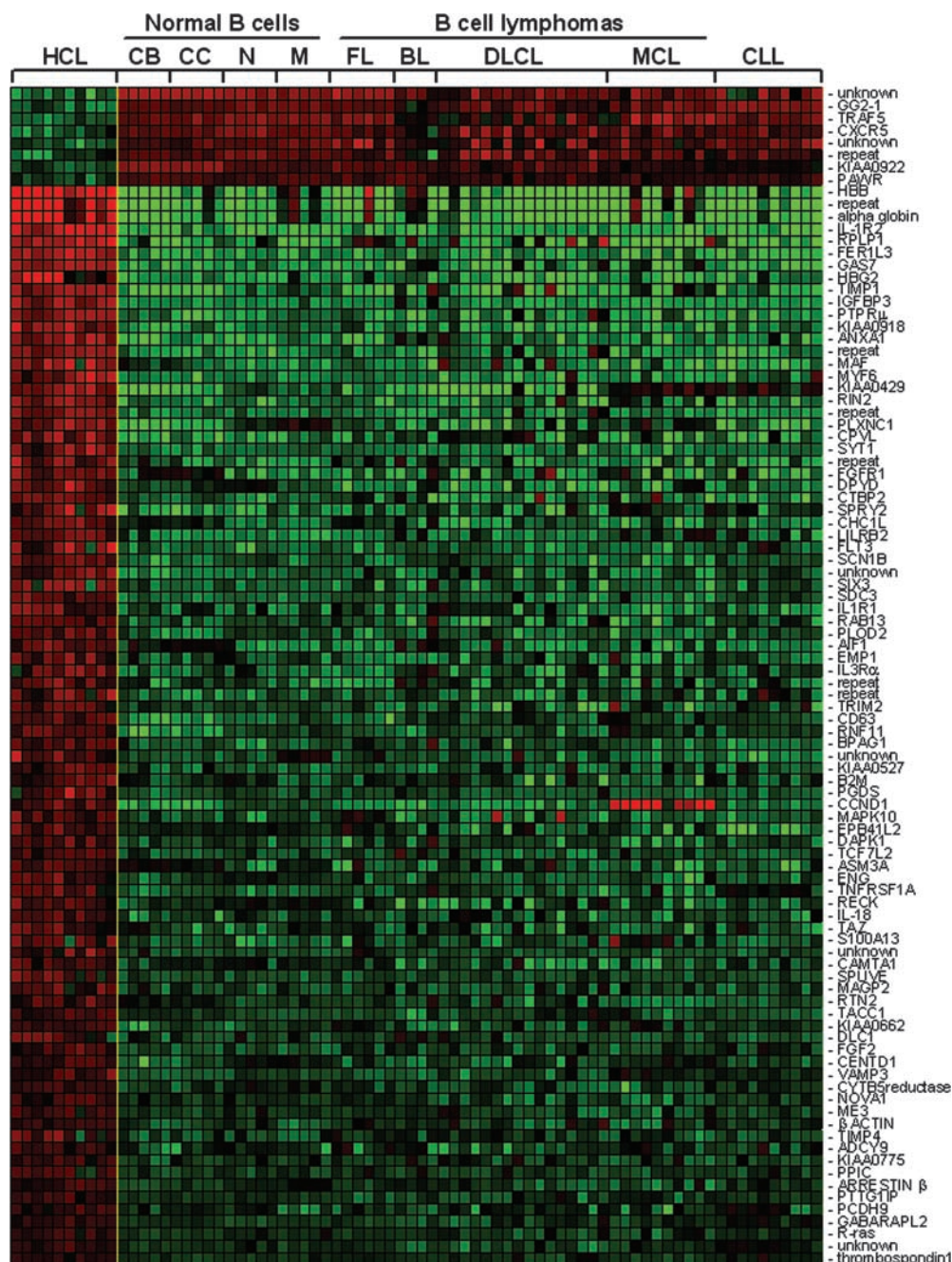


Figure 4. Identification of HCL-specific genes. A supervised analysis is performed using 10 samples of HCL (five BM biopsies and five non-BM samples) versus the normal B cell subpopulations (CB, CC, N, and M), different types of B-NHL (FL, BL, DLBCL, and MCL), and B-CLL. The B-NHL and B-CLL tumor samples are the same as those used in the unsupervised analysis (Fig. 1). The support value for the analysis was chosen as $n = n_o - 2$ (n_o , number of samples in the phenotype set). The analysis identified 89 genes that are differentially expressed in HCL versus all the other samples. Gene names are indicated, and some genes appear more than once (repeat). For GenBank/EMBL/DBJ accession nos. and Affymetrix codes, see Table S4 (available at <http://www.jem.org/cgi/content/full/jem.20031175/DC1>).

that are differentially expressed in HCL cells compared with the other samples (Fig. 4). Some of these genes were already known to be expressed in HCL (cyclin D1, FGF2, and IL-3R α ; references 26–28) and served as internal controls to validate our approach. We observed overexpression of cyclin D1 also in MCLs, as a reported hallmark of this type of lymphoma. Other known HCL markers (CD11c, CD68, CD85j, and CD103; references 29–32) were not identified by this type of analysis because their level of expression in HCL cells were not homogeneous and/or they were also expressed in other samples across the panel.

The expression of only seven genes was found decreased in HCL cells compared with all the other samples. These genes included the chemokine receptor CXCR5, involved in B cell homing (33), and the TNF receptor-associated factor 5, involved in the signal transduction of TNF-type receptors, including CD40 and CD27 (34).

Among the newly identified genes that are notably overexpressed in HCL are (also see Discussion): (a) GAS7, a growth arrest-specific (Gas) gene (35) that is essential for neurite outgrowth in cultured cerebellar neurons (overex-

pression of GAS7 in HCL may explain the characteristic projections of the hairy cells); (b) the FGFR1 receptor that, together with the previously reported overexpression of its ligand (FGF2; reference 27), suggests the presence in HCL of an autocrine loop; (c) the receptor tyrosine kinase FLT3 that is reported to function as a growth factor receptor for hematopoietic stem and/or progenitor cells (36); and (d) three different inhibitors of matrix metalloproteinases: TIMP1 and TIMP4, two secreted tissue inhibitors of metalloproteinases, and RECK, a membrane-anchored glycoprotein that represses synthesis and secretion of metalloproteinases (37).

Immunocytochemical Validation. To investigate whether the up-regulated mRNA levels of HCL-specific genes correspond to elevated levels of the corresponding proteins, we stained fresh cytopins and/or BM paraffin sections from patients with HCL (including the cases subjected to microarray and six additional HCL cases) using monoclonal and polyclonal antibodies directed against the proteins encoded by 22 of the HCL-specific genes.

The expression in HCL cells was confirmed for the following proteins: FGF2 (bFGF), annexin 1, CD135 (FLT3),

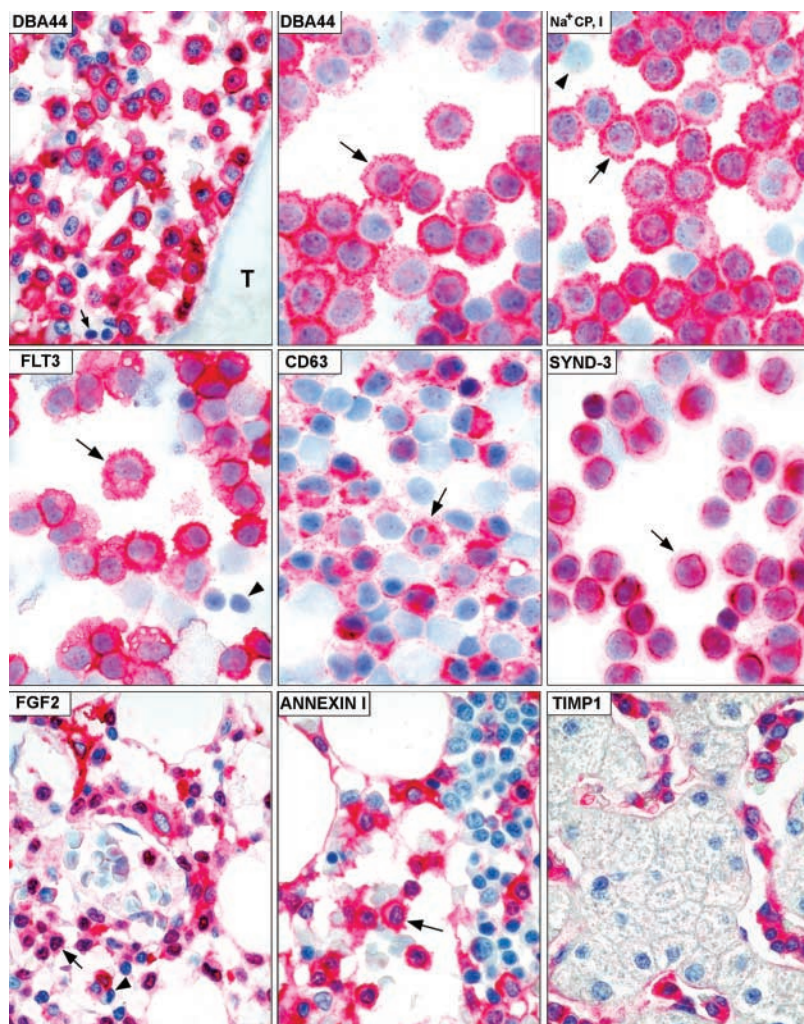


Figure 5. (top left, DBA44) BM biopsy from patient 5 showing massive infiltration by DBA44⁺ hairy cells; the arrow indicates rare residual hemopoietic precursors. (T) A bone trabecula (APAAP; 800 \times). (top middle, DBA44) Cytocentrifuge preparation (patient 3) showing strong surface positivity of HCL cells for DBA44 (arrow, APAAP; 800 \times). (Na^+ CP, I) Cytospin (patient 3). Positivity for Na^+ channel, type I, is mainly seen on the surface membrane (arrow). A negative lymphoid cell (arrowhead, APAAP; 800 \times). (FLT3) Cytospin (patient 3). Labeling of leukemic cells for FLT3 is mainly seen on the cell surface (arrow). A negative normal residual lymphocyte (arrowhead, APAAP; 800 \times). (CD63 and SYND-3) Cytospins (patient 3). Expression of CD63 and Syndecan-3 expression are mainly cytoplasmic (arrow, microgranular) and perinuclear (arrow), respectively (APAAP; 800 \times). (FGF2) BM biopsy (paraffin section) from patient 4. HCL cells show strong nuclear and cytoplasmic positivity for FGF2 (arrow, bFGF), whereas residual hemopoietic precursors are negative (arrowhead, APAAP; 800 \times). (Annexin I) BM biopsy (patient 4). Leukemic cells are strongly positive for annexin I (arrow). Positivity is mainly seen in the cytoplasm and surface, whereas nuclei are negative or faintly positive. An island of annexin 1-negative residual erythroid precursors is shown (APAAP; 800 \times). (TIMP1) Liver biopsy (paraffin section) from patient 3 showing typical infiltration of hepatic sinusoids by TIMP1⁺ leukemic hairy cells (APAAP; 800 \times).

Na⁺ CP type I (SCN1B), CD63, Syndecan-3, TIMP1 (Fig. 5) as well as IL-3R α , cyclin D1, FGFR1, GAS7, EPB4.1L2, β -actin, CPVL, β -arrestin-2, insulin-like growth factor binding protein (IGFBP3), MYF6 (Herculin), protein tyrosine phosphatase receptor μ (PTPR μ), Synaptotagmin 1, plexin-C1, TIMP4, and β -2-microglobulin (not depicted). Thus, these results indicate that the HCL-specific overexpression of genes detected by gene expression profiling is associated with the overexpression of the corresponding protein for all genes tested.

Discussion

This paper has addressed critical questions regarding the cell of origin and pathogenesis of HCL using gene expression profiling technology in a panel of 14 cases. This panel is relatively large considering the difficulties in collecting HCL samples suitable for gene expression profiling, due to the very limited availability of HCL cells from the BM (difficult extraction due to the reticulin fibers), from the peripheral blood (scarce presence of malignant cells), and from the spleen (decreasing use of the splenectomy as therapeutic approach; Materials and Methods). Nevertheless, the panel used here was sufficient to confirm the distinct phenotype of this disease, to identify its putative cell of origin, and to discover alterations in cellular programs that could be of biological and clinical relevance.

The Distinct Phenotype of HCL. HCL has been recognized for a long time as a chronic B cell malignancy with clinical, morphological, and phenotypic features clearly distinct from those of other types of B cell lymphomas (3, 4). The gene expression profile analysis performed here substantiates this concept by dramatically increasing the number of traits that characterize the unique HCL phenotype. In contrast to other B cell malignancies, where the gene expression profiling has identified unrecognized subsets of the disease (38), the phenotype of HCL appears to be homogeneous, suggesting that HCL is a single disease entity consistent with its homogeneous morphology and clinical behavior. The characteristic gene expression profile of HCL may be due to a distinct cellular origin and/or to a distinct mechanism of transformation.

The Cellular Derivation of HCL. Since its first description, the distinct HCL morphology and phenotype could not be assigned to a normal B cell developmental stage. The gene expression analysis shown here indicates that HCL is more related to memory B cells than to GCs or naive B cells. Recent findings that draw a relationship between a tonsillar B cell subset, identified by the phab V-3 antibody, and HCL (39) may be in accordance with the relationship between HCL cells and memory B cells we report here. In fact, phab V-3⁺ B cells do not express GC B cell markers and CD23, a marker of naive B cells, whereas they express the memory B cell marker CD27 and display mutated IgV sequences (39), thus, suggesting that they are memory B cells. As an additional similarity, the phab V-3⁺ tonsillar B cells and HCL cells are both

characterized by high level of expression of the CD11c molecule. Overall, the gene expression profiles together previous immunophenotypic observations suggest that HCL cells derive from memory B cells, possibly from a subset of this population characterized by phab V-3 and CD11c expression.

Pathogenetic Implications. The notion that HCL may derive from memory B cells has direct implications for the pathogenesis of this malignancy. HCL cells typically lack reciprocal balanced chromosomal translocations, which are the hallmark of all lymphoid malignancies, with the notable exception of B-CLLs (40). Intriguingly, B-CLLs also resemble memory B cells based on gene expression profiles (9). Thus, the only two lymphoid malignancies that lack chromosomal translocations are also both related to memory B cells. This observation further supports our previously proposed hypothesis that the lack of chromosomal translocations may be due to the fact the mechanism that can generate these aberrations (i.e., Ig VDJ recombination, class switching, and somatic hypermutation) are switched off in memory B cells (9). Thus, the pathogenesis of CLL and HCL may involve genetic lesions, such as the nonrandom deletions and amplifications found in these diseases that do not derive from mistakes of immunoglobulin genes remodeling mechanism.

Functional Alterations in HCL. This analysis provides several insights into the functional alterations of HCL. A careful comparison of HCL with memory B cells revealed a surprisingly conserved pattern of expression of genes that regulate functions typically damaged in malignant cells, such as proliferation and apoptosis. Similarly to memory B cells, HCL cells appear as largely quiescent cells expressing genes coding both anti- and proapoptotic molecules. Conversely, most of the differences between HCL and memory B cells involved the expression pattern of cytokines, chemokine receptors, and adhesion molecules.

Adhesion and Homing of HCL Cells. Several findings in this analysis may explain the unique dissemination pattern of HCL (i.e., involvement of BM, peripheral blood, and spleen red pulp with sparing of lymph nodes). The tendency of HCL to remain confined in blood-related compartments could be at least in part explained by the significant up-regulation of three genes (TIMP-1, TIMP-4, and RECK), all known to counteract the activity of matrix metalloproteinases, which promote local invasion, extravasation from blood vessels, and metastasis in all types of human neoplasms (41). Furthermore, the lack of spreading of HCL cells to lymph nodes can be explained by the down-regulation of CCR7 (sixfold compared with memory B cells), a chemokine receptor whose deficiency hampers the ability of B cells to enter lymph nodes (25). Consistent with previous immunocytochemical findings (42), our results show that HCL cells fail to express CXCR5, a chemokine receptor required for B cells homing to follicles of peripheral lymphoid organs (33).

Hairy Morphology. Although recognized as the hallmark of HCL, the “hairy” appearance of leukemic cells still

remains a feature for which no definitive explanation has been found. Interaction of the cytoskeleton-binding protein pp52 (LSP1) with F-actin-rich cytoskeletal arrays in the surface projections of HCL cells has been proposed as a mechanism for this property (43). Our results highlight other HCL-specific molecules that may contribute to the hairy phenotype, possibly interacting with β -actin (also up-regulated in HCL). These molecules are as follows: (a) Gas7 (Fig. 4), whose ectopic expression induces the formation of extended cellular processes and dramatic changes in cell shape by interacting with F-actin (44); and (b) EPB4.1L2 (Fig. 4), known to play a role in the maintenance of the shape of neuronal cells by interacting with β -actin underneath the plasma membrane (45).

Phagocytosis and Macrophage-like Features. It has been recognized for a long time that HCL cells have the capacity to phagocytose latex particles, zymosan, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (46, 47), leading to the hypothesis that HCL cells were either B lymphocytes with phagocytic capabilities (48) or malignant cells of monocytic origin (49). This phagocytic properties of HCL cells may be explained by specific up-regulation of annexin 1 (required for the phagocytic uptake of *Brucella suis* by human monocytes; reference 50), as well as CD63 (51) and CPVL (52), two macrophage-associated lysosomal molecules (Fig. 4). We speculate that phagocytosis and expression by HCL of several macrophage markers (CD11c, CD68, CD63, and CPVL) may be explained by the apparently abnormal up-regulation of c-Maf (Fig. 4), a transcription factor whose deregulated expression can induce macrophage differentiation (53, 54).

Marrow Fibrosis. Another distinctive feature of HCL is the fine reticulin marrow fibrosis (1–4), which is responsible for the frequent “dry-tap” at aspiration. This feature is likely due to the ability of HCL cells to synthesize and bind to a fibronectin matrix (55, 56), whose production rate is under control of the autocrine FGF2 secreted by HCL cells (57). The overexpression of IL-3R α and FLT3 by HCL cells (Fig. 4) might play a role in the fibrosis because the ligands for these two receptors (physiologically present in the BM stroma microenvironment) are important for adhesion of B cells to fibronectin through activation of the fibronectin receptors VLA4 and VLA5 (58), both known to be expressed in hairy cells (55, 59).

Clinical Implications. The results herein identify several antibodies that recognize HCL-specific molecules and, therefore, may be useful for the diagnosis of HCL. For instance, these reagents may help in the differential diagnosis between HCL and other borderline B cell chronic lymphoproliferative disorders (splenic lymphoma with villous lymphocytes), and in better defining the nature of the so-called HCL variant (60). These distinctions have therapeutic implications because HCL and splenic lymphoma with villous lymphocytes are sensitive to distinct therapeutic regimens (61). The selective sensitivity of HCL to IFN as compared with other B cell lymphomas may be explained by the up-regulation of the TNF receptor, TNFR1, which is likely to

potentiate the apoptotic effect of IFN- α -induced TNF- α secretion by HCL cells (62). Finally, HCL-specific cell surface molecules, such as IL-3R α , may represent potential targets for antibody-mediated immunotherapy, whereas the abnormal expression of the FLT3 tyrosine kinase receptor warrants further investigation on the sensitivity of HCL cells to the currently available inhibitors of FLT3.

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References

- Burke, J.S. 1978. The value of the bone-marrow biopsy in the diagnosis of hairy cell leukemia. *Am. J. Clin. Pathol.* 70: 876–884.
- Brunning, M., and R.W. McKenna. 1994. *Atlas of Tumor Pathology: Tumors of the Bone Marrow*. Armed Forces Institute of Pathology. Washington, D.C.: Universities Associated for Research and Education in Pathology.
- Harris, N.L., E.S. Jaffe, H. Stein, P.M. Banks, J.K. Chan, M.L. Cleary, G. Delsol, C. De Wolf-Peeters, B. Falini, and K.C. Gatter. 1994. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood*. 84:1361–1392.
- Jaffe, E.S., N.L. Harris, H. Stein, and J.W. Vardiman. 2001. World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. IARC Press, Lyon, France. 351 pp.
- Mey, U., J. Strehl, M. Gorschluter, C. Ziske, A. Glasmacher, H. Pralle, and I. Schmidt-Wolf. 2003. Advances in the treatment of hairy-cell leukaemia. *Lancet. Oncol.* 4:86–94.
- Maloum, K., C. Magnac, Z. Azgui, C. Cau, F. Charlotte, J.L. Binet, H. Merle-Beral, and G. Dighiero. 1998. VH gene expression in hairy cell leukaemia. *Br. J. Haematol.* 101:171–178.
- Forconi, F., S.S. Sahota, D. Raspadori, C.I. Mockridge, F. Lauria, and F.K. Stevenson. 2001. Tumor cells of hairy cell leukemia express multiple clonally related immunoglobulin isotypes via RNA splicing. *Blood*. 98:1174–1181.
- Rajewsky, K. 1996. Clonal selection and learning in the antibody system. *Nature*. 381:751–758.
- Klein, U., Y. Tu, G.A. Stolovitzky, M. Mattioli, G. Cattorini, H. Husson, A. Freedman, G. Inghirami, L. Cro, L. Baldini, et al. 2001. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J. Exp. Med.* 194:1625–1638.
- Klein, U., Y. Tu, G.A. Stolovitzky, J.L. Keller, J. Haddad,

- Jr., V. Miljkovic, G. Cattoretta, A. Califano, and R. Dalla-Favera. 2003. Transcriptional analysis of the B cell germinal center reaction. *Proc. Natl. Acad. Sci. USA*. 100:2639–2644.
11. Hartigan, J.A. 1975. Clustering Algorithms. John Wiley & Sons, Inc., New York. 351 pp.
 12. Eisen, M.B., P.T. Spellman, P.O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA*. 95:14863–14868.
 13. Califano, A., G. Stolovitzky, and Y. Tu. 2000. Analysis of gene expression microarrays for phenotype classification. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 8:75–85.
 14. Califano, A. 2000. SPLASH: structural pattern localization analysis by sequential histograms. *Bioinformatics*. 16:341–357.
 15. Falini, B., M. Fizzotti, A. Pucciarini, B. Bigerna, T. Marafioti, M. Gambacorta, R. Pacini, C. Alunni, L. Natali-Tanci, B. Ugolini, et al. 2000. A monoclonal antibody (MUM1p) detects expression of the MUM1/IRF4 protein in a subset of germinal center B cells, plasma cells, and activated T cells. *Blood*. 95:2084–2092.
 16. Cordell, J.L., B. Falini, W.N. Erber, A.K. Ghosh, Z. Abdulaziz, S. MacDonald, K.A. Pulford, H. Stein, and D.Y. Mason. 1984. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J. Histochem. Cytochem.* 32:219–229.
 17. Falini, B., M.F. Martelli, F. Tarallo, D.J. Moir, J.L. Cordell, K.C. Gatter, G. Loreti, H. Stein, and D.Y. Mason. 1984. Immunohistological analysis of human bone marrow trephine biopsies using monoclonal antibodies. *Br. J. Haematol.* 56: 365–386.
 18. Falini, B., D.Y. Mason, H. Stein, D.J. Moir, M. Zuccaccia, F. Grignani, and M.F. Martelli. 1984. The immunohistological analysis of undecalcified human bone marrow trephine biopsies. Technical and diagnostic aspects. *Haematologica*. 69: 346–376.
 19. Pascual, V., Y.J. Liu, and J. Banchereau. 1997. Normal human B cell sub-populations and their malignant counterparts. *Baillieres Clin. Haematol.* 10:525–538.
 20. Kuppers, R., U. Klein, M.L. Hansmann, and K. Rajewsky. 1999. Cellular origin of human B-cell lymphomas. *N. Engl. J. Med.* 341:1520–1529.
 21. Falini, B., R. Schwarting, W. Erber, D.N. Posnett, M.F. Martelli, F. Grignani, M. Zuccaccia, K.C. Gatter, C. Cernetti, H. Stein, et al. 1985. The differential diagnosis of hairy cell leukemia with a panel of monoclonal antibodies. *Am. J. Clin. Pathol.* 83:289–300.
 22. Flenghi, L., B. Bigerna, M. Fizzotti, S. Venturi, L. Pasqualucci, S. Pileri, B.H. Ye, M. Gambacorta, R. Pacini, C.D. Baroni, et al. 1996. Monoclonal antibodies PG-B6a and PG-B6p recognize, respectively, a highly conserved and a formol-resistant epitope on the human BCL-6 protein amino-terminal region. *Am. J. Pathol.* 148:1543–1555.
 23. Klein, U., K. Rajewsky, and R. Kuppers. 1998. Human immunoglobulin (Ig)M⁺IgD⁺ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J. Exp. Med.* 188:1679–1689.
 24. Kong, Y.Y., H. Yoshida, I. Sarosi, H.L. Tan, E. Timms, C. Capparelli, S. Morony, A.J. Oliveira-dos-Santos, G. Van, A. Itie, et al. 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature*. 397:315–323.
 25. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. 99: 23–33.
 26. Bosch, F., E. Campo, P. Jares, S. Pittaluga, J. Munoz, I. Nayach, M.A. Piris, C. Dewolf-Peeters, E.S. Jaffe, C. Rozman, et al. 1995. Increased expression of the PRAD-1/CCND1 gene in hairy cell leukaemia. *Br. J. Haematol.* 91:1025–1030.
 27. Gruber, G., J.D. Schwarzmeier, M. Shehata, M. Hilgarth, and R. Berger. 1999. Basic fibroblast growth factor is expressed by CD19/CD11c-positive cells in hairy cell leukemia. *Blood*. 94:1077–1085.
 28. Munoz, L., J.F. Nomdedeu, O. Lopez, M.J. Camicer, M. Bellido, A. Aventin, S. Brunet, and J. Sierra. 2001. Interleukin-3 receptor alpha chain (CD123) is widely expressed in hematologic malignancies. *Haematologica*. 86:1261–1269.
 29. Falini, B., K. Pulford, W.N. Erber, D.N. Posnett, G. Pallen, R. Schwarting, L. Annino, A. Cafolla, S. Canino, A. Mori, et al. 1986. Use of a panel of monoclonal antibodies for the diagnosis of hairy cell leukaemia. An immunocytochemical study of 36 cases. *Histopathology*. 10:671–687.
 30. Falini, B., L. Flenghi, S. Pileri, M. Gambacorta, B. Bigerna, H. Durkop, F. Eitelbach, J. Thiele, R. Pacini, A. Cavaliere, et al. 1993. PG-M1: a new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. *Am. J. Pathol.* 142: 1359–1372.
 31. Banham, A.H., M. Colonna, M. Cella, K.J. Micklem, K. Pulford, A.C. Willis, and D.Y. Mason. 1999. Identification of the CD85 antigen as ILT2, an inhibitory MHC class I receptor of the immunoglobulin superfamily. *J. Leukoc. Biol.* 65:841–845.
 32. Flenghi, L., F. Spinozzi, H. Stein, M. Krushwitz, S. Pileri, and B. Falini. 1990. LF61: a new monoclonal antibody directed against a trimeric molecule (150 kDa, 125 kDa, 105 kDa) associated with hairy cell leukaemia. *Br. J. Haematol.* 76: 451–459.
 33. Forster, R., A.E. Mattis, E. Kremmer, E. Wolf, G. Brem, and M. Lipp. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell*. 87:1037–1047.
 34. Nakano, H., S. Sakon, H. Koseki, T. Takemori, K. Tada, M. Matsumoto, E. Munechika, T. Sakai, T. Shirasawa, H. Akiba, et al. 1999. Targeted disruption of Traf5 gene causes defects in CD40- and CD27-mediated lymphocyte activation. *Proc. Natl. Acad. Sci. USA*. 96:9803–9808.
 35. Ju, Y.T., A.C. Chang, B.R. She, M.L. Tsaur, H.M. Hwang, C.C. Chao, S.N. Cohen, and S. Lin-Chao. 1998. gas7: A gene expressed preferentially in growth-arrested fibroblasts and terminally differentiated Purkinje neurons affects neurite formation. *Proc. Natl. Acad. Sci. USA*. 95:11423–11428.
 36. Maroc, N., R. Rottapel, O. Rosnet, S. Marchetto, C. Lavezzi, P. Mannoni, D. Birnbaum, and P. Dubreuil. 1993. Biochemical characterization and analysis of the transforming potential of the FLT3/FLK2 receptor tyrosine kinase. *Oncogene*. 8:909–918.
 37. Oh, J., R. Takahashi, S. Kondo, A. Mizoguchi, E. Adachi, R.M. Sasahara, S. Nishimura, Y. Imamura, H. Kitayama, D.B. Alexander, et al. 2001. The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell*. 107:789–800.
 38. Alizadeh, A.A., M.B. Eisen, R.E. Davis, C. Ma, I.S. Lossos, A. Rosenwald, J.C. Boldrick, H. Sabet, T. Tran, X. Yu, et

- al. 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*. 403:503–511.
39. van Der Vuurst De Vries, A.R., and T. Logtenberg. 1999. A phage antibody identifying an 80-kDa membrane glycoprotein exclusively expressed on a subpopulation of activated B cells and hairy cell leukemia B cells. *Eur. J. Immunol.* 29:3898–3907.
 40. Tam, W., and R. Dalla-Favera. 2001. Proto-oncogenes and tumor suppressor genes in hematopoietic malignancies. In *Neoplastic Hematopathology*. D.M. Knowles, editor. Lip-pincott Williams & Wilkins, New York. 329–363.
 41. Yoon, S.O., S.J. Park, C.H. Yun, and A.S. Chung. 2003. Roles of matrix metalloproteinases in tumor metastasis and angiogenesis. *J. Biochem. Mol. Biol.* 36:128–137.
 42. Durig, J., U. Schmucker, and U. Dührsen. 2001. Differential expression of chemokine receptors in B cell malignancies. *Leukemia*. 15:752–756.
 43. Miyoshi, E.K., P.L. Stewart, P.W. Kincade, M.B. Lee, A.A. Thompson, and R. Wall. 2001. Aberrant expression and localization of the cytoskeleton-binding pp52 (LSP1) protein in hairy cell leukemia. *Leuk. Res.* 25:57–67.
 44. She, B.R., G.G. Liou, and S. Lin-Chao. 2002. Association of the growth-arrest-specific protein Gas7 with F-actin induces reorganization of microfilaments and promotes membrane outgrowth. *Exp. Cell Res.* 273:34–44.
 45. Kontogianni-Konstantopoulos, A., C.S. Frye, E.J. Benz, Jr., and S.C. Huang. 2001. The prototypical 4.1R–10-kDa domain and the 4.1g–10-kDa paralog mediate fodrin-actin complex formation. *J. Biol. Chem.* 276:20679–20687.
 46. Jansen, J., C.J. Meijer, P. van der Valk, W.C. de Bruyn, P.C. Leijh, G.J. den Ottolander, and R. van Furth. 1979. Phagocytic potential of hairy cells. *Scand. J. Haematol.* 23:69–79.
 47. Rosner, M.C., and H.M. Golomb. 1982. Phagocytic capacity of hairy cells from seventeen patients. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* 40:327–337.
 48. Utsinger, P.D., W.J. Yount, C.R. Fuller, M.J. Logue, and E.P. Orringer. 1977. Hairy cell leukemia: B-lymphocyte and phagocytic properties. *Blood*. 49:19–27.
 49. Scheinberg, M., A.I. Brenner, A.L. Sullivan, E.S. Cathcart, and I. Katayama. 1976. The heterogeneity of leukemic reticuloendotheliosis, “hairy cell leukemia.” Evidence for its monocytic origin. *Cancer*. 37:1302–1307.
 50. Kusumawati, A., C. Cazevielle, F. Porte, S. Bettache, J.P. Liautard, and J. Sri Widada. 2000. Early events and implication of F-actin and annexin I associated structures in the phagocytic uptake of *Brucella suis* by the J-774A.1 murine cell line and human monocytes. *Microb. Pathog.* 28:343–352.
 51. Fukuda, M. 1991. Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *J. Biol. Chem.* 266:21327–21330.
 52. Mahoney, J.A., B. Ntoli, R.P. DaSilva, S. Gordon, and A.J. McKnight. 2001. Cloning and characterization of CPVL, a novel serine carboxypeptidase, from human macrophages. *Genomics*. 72:243–251.
 53. Hedge, S.P., A. Kumar, C. Kurschner, and L.H. Shapiro. 1998. c-Maf interacts with c-Myb to regulate transcription of an early myeloid gene during differentiation. *Mol. Cell. Biol.* 18:2729–2737.
 54. Hegde, S.P., J. Zhao, R.A. Ashmun, and L.H. Shapiro. 1999. c-Maf induces monocytic differentiation and apoptosis in bi-potent myeloid progenitors. *Blood*. 94:1578–1589.
 55. Burthem, J., and J.C. Cawley. 1994. The bone marrow fibrosis of hairy-cell leukemia is caused by the synthesis and assembly of a fibronectin matrix by the hairy cells. *Blood*. 83:497–504.
 56. Aziz, K.A., K.J. Till, M. Zuzel, and J.C. Cawley. 2000. Involvement of CD44-hyaluronan interaction in malignant cell homing and fibronectin synthesis in hairy cell leukemia. *Blood*. 96:3161–3167.
 57. Aziz, K.A., K.J. Till, H. Chen, J.R. Slupsky, F. Campbell, J.C. Cawley, and M. Zuzel. 2003. The role of autocrine FGF-2 in the distinctive bone marrow fibrosis of hairy-cell leukaemia (HCL). *Blood*. 10:10.
 58. Shibayama, H., N. Anzai, A. Ritchie, S. Zhang, C. Mantel, and H.E. Broxmeyer. 1998. Interleukin-3 and Flt3-ligand induce adhesion of Baf3/Flt3 precursor B-lymphoid cells to fibronectin via activation of VLA-4 and VLA-5. *Cell. Immunol.* 187:27–33.
 59. Angelopoulou, M.K., F.N. Kontopidou, and G.A. Pangalis. 1999. Adhesion molecules in B-chronic lymphoproliferative disorders. *Semin. Hematol.* 36:178–197.
 60. Sainati, L., E. Matutes, S. Mulligan, M.P. de Oliveira, S. Rani, I.A. Lampert, and D. Catovsky. 1990. A variant form of hairy cell leukemia resistant to alpha-interferon: clinical and phenotypic characteristics of 17 patients. *Blood*. 76:157–162.
 61. Hermine, O., F. Lefrere, J.P. Bronowicki, X. Mariette, K. Jondeau, V. Eclache-Saudreau, B. Delmas, F. Valensi, P. Caccoub, C. Brechot, et al. 2002. Regression of splenic lymphoma with villous lymphocytes after treatment of hepatitis C virus infection. *N. Engl. J. Med.* 347:89–94.
 62. Baker, P.K., A.R. Pettitt, J.R. Slupsky, H.J. Chen, M.A. Glenn, M. Zuzel, and J.C. Cawley. 2002. Response of hairy cells to IFN-alpha involves induction of apoptosis through autocrine TNF-alpha and protection by adhesion. *Blood*. 100: 647–653.