Familial B-Cell Chronic Lymphocytic Leukemia

Analysis of Cytogenetic Abnormalities, Immunophenotypic Profiles, and Immunoglobulin Heavy Chain Gene Usage

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Abstract

B-cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous disease that may exhibit familial clustering. We examined the cytogenetic, immunophenotypic, and VH gene usage characteristics of a family with B-CLL affecting 7 members in 3 generations. Interphase fluorescence in situ hybridization studies identified an acquired deletion of chromosome 13q14 in the leukemic cells of 6 affected members, accompanied by deletion 14q32 or trisomy 12 in 2 cases. VH gene analysis demonstrated clonal rearrangements of the VH3 gene family in 5 cases and of VH2 genes in 1 case. Two cases had a second VH1 family gene rearrangement that was unmutated. Flow cytometry performed on 5 cases showed the typical B-CLL immunophenotype; all were CD38−, but 3 expressed ZAP-70. Our findings support previous observations that familial and sporadic B-CLL cases are biologically similar and suggest that familial clusters will be useful for studying pathogenetic events in B-CLL.
of studies have compared the biologic features of sporadic and familial cases. For example, few studies have systematically examined familial clusters of B-CLL using fluorescence in situ hybridization (FISH) to demonstrate recurrent cytogenetic abnormalities commonly seen in sporadic B-CLL. Likewise, preliminary studies have suggested that overexpression of CD38 and ZAP-70 occurs at similar rates and that there is preferential usage of the \( V_H1 \), \( V_H3 \), and \( V_H4 \) gene families. The frequency at which specific genes are used is more difficult to ascertain owing to the limited number of cases studied.

We have previously described 3 generations of a family in which the father and all 4 male offspring, including twin brothers, were affected by B-CLL. Conventional and molecular cytogenetic analysis of peripheral blood samples demonstrated the presence of an acquired del13q14 in the leukemic cells of all 4 affected offspring, without constitutional cytogenetic abnormalities.

In the present study, we used conventional and FISH cytogenetics and immunophenotypic studies to determine if additional asymptomatic family members were affected in this family. We also extended our previous FISH cytogenetic studies with additional probes and determined the mutational status, \( V_H \) gene usage, and ZAP-70 expression of the B-CLL cells from affected family members to correlate these findings with each other and with the FISH cytogenetic results.

**Materials and Methods**

**Patients and Samples**

A family in which the father and all 4 male children were affected by B-CLL has been previously described. For the present study, additional family members were identified in 3 generations, for a total of 7 affected people. The

![Figure 1](image-url) Pedigree of a family in which multiple members of 3 generations have been affected by B-cell chronic lymphocytic leukemia (B-CLL). The pattern is consistent with an autosomal dominant genetic predisposition to B-CLL. The decreasing age of onset across generations suggests anticipation. Co, colon cancer; CSU, cancer site unknown; Pro, prostate cancer. (Pedigree drawn by Tami Richardson-Nelson, BGS.)
diagnosis of B-CLL was confirmed by review of the morphologic, immunophenotypic, cytogenetic, and/or clinical materials. A detailed hematologic and solid tumor family history was obtained from the proband (Figure 1, IV:4). Additional family members were contacted, and consent was obtained from each family member entering the study. Peripheral blood, skin biopsy, and buccal mucosa cytology samples were obtained for analysis from 6 affected and 9 unaffected family members. This study was reviewed and approved by the institutional review board at the Creighton University School of Medicine, Omaha, NE.

**Cytogenetic Studies**

Peripheral blood samples from 15 family members (6 affected and 9 unaffected) were studied by conventional and/or molecular cytogenetic techniques by FISH. Additional FISH studies were also performed on buccal mucosal samples from 2 affected members with multiple FISH abnormalities to determine whether these were constitutional or acquired. For conventional cytogenetic studies, blood samples were cultured with and without mitogen stimulation for 24 and 72 hours using standard techniques, and 20 G-banded metaphases were analyzed from each sample. Interphase FISH studies using probes to chromosomes 12, 13q14/13q34, and 14q32; the ATM locus on 11q23; and the p53 locus on 17p13.1 (all from Vysis, Downers Grove, IL) with hybridization on a HyBrite instrument (Vysis) were performed. At least 100 nuclei were examined for each probe. The false-positive and false-negative cutoff values used for these probes were those established by the Human Genetics Laboratory, University of Nebraska Medical Center in clinical studies of more than 500 analyses with these probes.

**Flow Cytometric Immunophenotyping**

Peripheral blood samples were obtained for immunophenotyping from 5 affected (Figure 1, IV:1, IV:2, IV:4, IV:9, and V:4) and 7 unaffected (Figure 1, V:1, V:2, V:3, V:6, V:7, V:7, V:8) family members. For flow cytometry studies, peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated peripheral blood by density gradient centrifugation (Accu-Prep, Accurate Chemical, Westbury, NY) and hypotonic lysis. PBMCs were then washed with phosphate-buffered saline and resuspended in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 1 mol/L of Hepes buffer, L-glutamine, penicillin, streptomycin, and 10% fetal calf serum for immediate analysis or in Cell Culture Freezing Media with 10% dimethyl sulfoxide (GIBCO BRL/Invitrogen, Rockville, MD) for storage in liquid nitrogen.

Isolated PBMCs were then labeled with antibodies to CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11c, CD16, CD19, CD20, CD23, CD24, CD25, CD38, CD45, CD56, CD103, and HLA-DR for multicolor flow cytometry studies (Beckman Coulter EPICS XL or FC500 flow cytometer, Beckman Coulter, Miami, FL). B-CLL populations were further studied for cytoplasmic ZAP-70 expression using fresh or thawed sample aliquots, FIX AND PERM Cell Permeabilization reagents (Caltag Laboratories, Burlingame, CA), and 2 different ZAP-70 antibody clones (clone IE7.2, Caltag Laboratories; clone 2F3.2, Upstate Laboratories, Charlottesville, VA). ZAP-70 expression by B-CLL cells was determined using a 20% positive cutoff and a T-cell threshold, as previously described.

**\( V_H \) Gene Usage and Mutation Analysis**

\( V_H \) gene usage and mutation analysis was performed on PBMCs from 6 affected family members (Figure 1, IV:1-4, IV:9, and V:4). DNA was isolated from fresh or frozen PBMCs after standard Proteinase K digestion, followed by phenol-chloroform extraction. Total cellular RNA was isolated from fresh PBMCs by Trizol reagent (Invitrogen, Carlsbad, CA). After DNase I (Promega, Madison, WI) treatment, 1 µg of total RNA was reverse transcribed to complementary DNA with random hexamers using Superscript II reverse transcriptase (Invitrogen). Rearranged \( V_H \) genes were then amplified from extracted DNA by polymerase chain reaction (PCR) using \( V_H \) family–specific primers to framework 1 (FR1) sequences and a consensus sequence at the 3' end of the J region (J\( _H \) external) in a 50-µL volume, using a Biometra thermocycler (Biometra, Kent, England). Primer sequences are shown in Table 1. The PCR reaction contained a final concentration of 1× PCR buffer with 0.2 mmol/L of each primer, 1.5 mmol/L of magnesium chloride (for \( V_H^1, V_H^2, \) and \( V_H^4 \)) or 2.0 mmol/L of magnesium chloride (for \( V_H^2, V_H^5, \) and \( V_H^6 \)), 0.2 mmol/L of each deoxynucleoside triphosphate, and 2.0 U of Taq DNA polymerase (Promega).

Thirty-five cycles of PCR were performed using the following cycling conditions after an initial incubation at 94°C for 5 minutes: denaturing at 94°C for 40 seconds, annealing at 65°C for 40 seconds, and extension at 72°C for 1 minute. In cases in which 2 rearranged \( V_H \) genes were identified, reverse transcriptase–PCR was also performed for these samples using the same PCR conditions except for an initial denaturing step of 2 minutes instead of 5 minutes. For these reactions, the leader primers of the \( V_H^1 \) and \( V_H^2 \) families replaced the FR1 primer.

The PCR products were analyzed on 10% Precast TBE Gel (Bio-Rad, Hercules, CA) and visualized by ethidium bromide staining. The bands were excised, and the DNA was extracted by freeze thawing the gel slice (1 gel slice in 200 µL of sterile water) 3 times using a –70°C freezer. To prepare for sequencing, 2 µL of the extracted DNA was reamplified using the \( V_H \) family–specific primer and M13RJH nested primers for 25 cycles. The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Santa Clarita, CA) and sent.
directly for sequencing using the family-specific FR1 primer or M13-reverse primer for sequencing reactions. Sequencing runs were performed on a Beckman Coulter CEQ 2000 XL8 capillary DNA sequencer (Beckman Coulter) using dye-terminator chemistry.

The sequences obtained from each sample were compared with previously published germline \( V_H, D_H, \) and \( J_H \) segment sequences in the V Base sequence directory (http://www.mrc-cpe.cam.ac.uk/vbase), and the closest germline sequence was assigned. If 2% or more of the \( V_H \) gene sequence differed from the germline sequence, the sample was classified as mutated.

**Results**

**Clinical Features**

B-CLL was diagnosed clinically in 7 family members (Figure 1), including the affected father (Figure 1, III:1), his 4 sons (Figure 1, monozygotic twins IV:1 and IV:2, IV:3, and IV:4), and a son of 1 of the twin brothers (Figure 1, V:4). The only known female case (Figure 1, IV:9) is a cousin of the proband. The median age at diagnosis was 55 years (range, 34-77 years). The affected father was deceased at the time of the study. Absolute lymphocyte counts at the time of diagnosis were available for 4 of 7 affected people and ranged from \( 7.0 \times 10^9/L \) (case IV:1) to \( 164.9 \times 10^9/L \) (case III:1). Of the 6 living affected members, 5 had received treatment for B-CLL at the time of the study. In addition to B-CLL, 1 of the 4 brothers (Figure 1, IV:3) also had prostate carcinoma. A detailed family history indicated other unaffected family members with prostate (Figure 1, III:7) or colon (Figure 1, III:6) carcinoma and 1 additional family member with a malignancy of unknown type (Figure 1, II:3).

**Cytogenetic Features**

The del13q14 was identified by interphase FISH in peripheral blood samples from all 6 affected family members alive at the time of the study and was the sole abnormality in 4 of 6 cases (Table 2). In 1 patient (IV:3), interphase FISH cytogenetic studies demonstrated deletion of both copies of...
chromosome 13q14 (nullisomy) in 68% of the nuclei and deletion of 1 copy of chromosome 13q14 in 9% of the nuclei. Conventional cytogenetic studies following 72-hour cultures with mitogen stimulation of this patient’s peripheral blood sample demonstrated a normal karyotype, and FISH studies for chromosome 13q14 performed on buccal mucosa were normal, confirming that del13q14 was an acquired cytogenetic abnormality. Repeated interphase FISH studies performed on a peripheral blood sample obtained 4 years later demonstrated nullisomy of 13q14 in 80% of nuclei, del13q14 in 17% of nuclei, and an additional deletion of the p53 locus at 17p13.1 in 73% of nuclei, consistent with cytogenetic progression.

The del13q14 was accompanied by a deletion of 14q32 in 1 case (Figure 1, IV:2) and trisomy 12 (Figure 1, IV:9) in another. Interphase FISH studies for case IV:9 showed trisomy 12 and del13q14 abnormalities present simultaneously in the majority of nuclei. Conventional cytogenetic studies of peripheral blood samples from both of these patients yielded normal karyotypes. Interphase FISH studies for abnormalities of 13q14 and 14q32 performed on buccal mucosa cells from case IV:2 were negative for abnormalities of either region. Additional skin or buccal mucosa samples were not available for case IV:9.

Conventional cytogenetic (5 cases) and/or interphase FISH (9 cases) studies performed on peripheral blood samples from 9 unaffected family members did not demonstrate any constitutional or acquired abnormalities.

**Immunophenotypic Features**

Immunophenotyping by flow cytometry was performed on peripheral blood samples from 5 of 6 living affected members and showed the typical CD5+, CD19+, dim CD20+, and CD23+ pattern of B-CLL, with low- or intermediate-density monotypic κ (2/5) or λ (3/5) light chain expression. All 5 cases were negative for CD38. However, 3 of the 5 were positive for ZAP-70 (Table 2). Of the 5 cases, 2 had low-density (dim) CD5 expression, and the other 3 showed moderately intense expression. Neither ZAP-70 expression nor intensity of CD5 expression correlated with mutational status.

Immunophenotyping by flow cytometry was also performed in 7 of the 9 clinically unaffected family members participating in the study. None of these family members had an absolute or relative lymphocytosis at the time of the study. No subclinical, CD5+, monoclonal B-cell populations were identified among these clinically unaffected family members.

**Immunoglobulin Gene Usage and Heavy Chain Variable Region Mutation Status**

For 6 affected members, material was available for VH mutation analysis. The mutation data are summarized in Table 3. Sequencing of the PCR products demonstrated a single clonally rearranged VH gene in 4 of 6 cases. In the other 2 cases, 2 clonally rearranged VH genes were identified. The VH3 gene family was expressed in 5 of 6 cases, and VH2 was expressed in 1 case. In 3 of 5 cases using the VH3 family, the VH3-7 gene was rearranged. Both cases having 2 VH gene rearrangements used the VH3 and VH1 families. All 6 cases were classified as mutated in VH2 or VH3. In the 2 cases with 2 VH rearrangements, the VH1 family genes were unmutated. In both of these cases, expression of 2 in-frame rearrangements was confirmed by reverse transcriptase–PCR, suggesting the presence of 2 different clones in each sample. However, analysis of the phenotypic characteristics of the leukemic cells from these cases did not clearly identify 2 clonal populations, and the intensity of CD5 expression was uniform in both cases.

**Discussion**

The del13q14 is the most common genetic abnormality found in B-CLL when tested by FISH methods and is associated with a good prognosis when present as a sole abnormality. The presence of an acquired del13q14 in tumor cells from all members of this family affected by B-CLL, including identical twins, strongly suggests an inherited predisposition to developing B-CLL. The pattern of inheritance in this family seems to be autosomal dominant, and the identification of an affected cousin (IV:9) is consistent with incomplete

| TABLE 3 |
| Mutation Analysis in Family Members With B-Cell Chronic Lymphocytic Leukemia |
| Case No. | VH Family | VH Gene | % Identity | DH Gene | JH Gene |
| IV:1 | VH3 | 3-11 | 96.0 | D1-14 | J4 |
| IV:2 | VH1 | 1-46 | 100.0 | D3-22 | J4 |
| IV:3 | VH3 | 3-15 | 97.0 | D6-13 | J4 |
| IV:4 | VH3 | 3-7 | 95.0 | D5-5 | J4 |
| IV:9 | VH3 | 3-7 | 96.0 | D6-13 | J6 |
| V:4 | VH3 | 3-7 | 95.0 | D3-22 | J4 |

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penetration. Because the father and his 4 sons lived on a farm, an alternative explanation for this familial cluster would be a common environmental exposure. However, environmental risk factors have not been clearly identified in sporadic or familial B-CLL, although a small increase in risk of developing B-CLL has been associated with farming and has been attributed to exposure to certain pesticides (reviewed by Caporaso et al). In this family, neither the offspring of one of the brothers, case V:4, nor the affected cousin, case IV:9, grew up on the same farm as the 4 brothers. This latter finding also supports the hypothesis that an inherited abnormality, rather than a common environmental exposure, had an important role in this familial disease cluster.

To our knowledge, only 3 other studies have used FISH to systematically examine familial B-CLL cases for the presence of recurrent cytogenetic abnormalities commonly identified in sporadic cases. Summersgill et al identified a sibling pair with trisomy 12, and Espinet et al identified 2 brothers with different abnormal karyotypes by conventional studies but who shared del13q14 as shown by FISH studies. Mehes et al described 2 brothers with diploid Y chromosomes by FISH studies and Y chromosome gains by comparative genomic hybridization; 1 of the brothers was also positive for del13q14. Our findings, in conjunction with these reports, support the hypothesis that familial and sporadic B-CLL cases undergo similar genetic events during pathogenesis. A recent study of cytogenetic aberrations in sporadic cases of B-CLL found that in some patients, del13q14 was present in only a subset of purified CD5+CD19+ cells, suggesting that in some patients, del13q14 was present in only a subset of purified CD5+CD19+ cells, suggesting that these abnormalities are clonal events rather than a common environmental exposure. Therefore, the hypothesis that an inherited abnormality, rather than a common environmental exposure, had an important role in this familial disease cluster.

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expressed in-frame. It is unlikely that a single B-cell clone would express 2 V_H genes. More likely, 2 clones expressing different V_H genes were present in each of these samples. For each of the 2 cases with 2 clonal rearrangements, both rearrangements were sequenced and shown to be unrelated (Table 3).

The molecular cytogenetic abnormalities, pattern of immunoglobulin gene usage and mutation, and the immunophenotypic findings in this family are similar to those seen in sporadic B-CLL cases. Our findings support previous observations that familial cases of B-CLL demonstrate similarities to sporadic cases, and, therefore, familial clusters are likely to be useful for studying predisposing and initiating genetic events in B-CLL.

References


