Molecular Genetic Demonstration of the Diverse Evolution of Richter’s Syndrome (Chronic Lymphocytic Leukemia and Subsequent Large Cell Lymphoma)

By András Matolcsy, Giorgio Inghirami, and Daniel M. Knowles

Paired samples of chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL) and the subsequent diffuse large cell lymphoma (DLL) of six cases of Richter’s syndrome were investigated to establish the clonal relationship between the CLL/SLL and the DLL components and to define the oncogene and/or tumor-suppressor gene alterations involved in the morphologic transformation of CLL/SLL. Southern blot hybridization analysis showed identical clonal immunoglobulin (Ig) gene-rearrangement patterns in the CLL/SLL and DLL components in four cases and different Ig gene-rearrangement patterns in two cases. Polymerase chain reaction (PCR) amplification, cloning, and DNA sequencing of complementary determinant region 3 (CDR3) of the Ig-heavy chain gene of one of the two cases in which the Ig gene-rearrangement patterns were different showed nonidentical sequences in the CLL/SLL and DLL components. In the other case, monomorphic Epstein-Barr virus (EBV) genome integration was detected in the DLL but not in the CLL, suggesting that the CLL and DLL components in this case of Richter’s syndrome also represent unrelated clones. Single-strand conformation polymorphism (SSCP) analysis and sequencing of exons 5 and 9 of the p53 tumor-suppressor gene showed a mutation in codon 176 of the DLL but not in the CLL/SLL component in one case where the CLL/SLL and DLL represented different clones. The p53 mutation probably played a role in the development of the lymphoma rather than morphologic transformation of the CLL/SLL in this case. SSCP analysis and sequencing also showed identical mutations in codon 282 in both the CLL/SLL and DLL components in a case where the DLL represented identical clones. Thus, this p53 gene mutation was present both before and after morphologic transformation, and therefore, probably did not play a primary role in this process. Southern blot hybridization analysis failed to show evidence of bcl-1, bcl-2, c-myc proto-oncogene or retinoblastoma (Rb) tumor-suppressor gene rearrangements in these six cases of Richter’s syndrome. In conclusion, the original CLL/SLL and the subsequent DLL in Richter’s syndrome may or may not be derived from identical clones, and the well-known proto-oncogenes and tumor-suppressor genes do not appear to play an obvious and consistent role in the morphologic transformation of CLL/SLL to DLL.

A P P R O X I M A T E L Y 3% to 5% of cases of chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL) show evidence of morphologic transformation to diffuse large cell lymphoma (DLL). This process is commonly referred to as Richter’s syndrome. It is usually accompanied by marked clinical progression of the disease and a fatal outcome. However, despite the well-known occurrence of this phenomenon, the precise clonal relationship between the two neoplastic cell populations is still unclear. It has been suggested, based on immunohistologic, immunoglobulin (Ig) isotype, anti-idiotypic, Ig gene rearrangement and cytogenetic studies, that the DLL develops as a clonal evolution from CLL/SLL, or alternatively, that the two neoplasms are distinct, unrelated clonal proliferations. Unfortunately, a definitive conclusion cannot be drawn from these conflicting reports. In fact, there are many potential explanations for these disparate results. For example, different cases have been evaluated by different methods, each of which possess their own limitations. Furthermore, somatic point mutation, isotype switching and postarrangement gene deletion of the Ig genes may be responsible for observed differences in Southern blot analyses and different reactivities of anti-idiotypic antibodies.

Oncogene activation and tumor-suppressor gene mutation are not known to occur in the majority of cases of CLL/SLL, although bcl-1 and bcl-2 rearrangements and p53 gene mutations have been reported in sporadic cases. In contrast, elevated c-myc oncogene expression, retinoblastoma (Rb) gene deletion, and p53 tumor-suppressor gene mutation have been reported in some cases of CLL/SLL showing clinical progression and morphologic transformation. These findings suggest that certain specific genetic events may play a role in this transformation. However, comparative studies of structural alterations of oncogenes and tumor-suppressor genes in both the CLL/SLL and the DLL components occurring in the same patient have not been performed.

For these reasons, we sought to determine the clonal relationship between the original CLL/SLL and the subsequent DLL in Richter’s syndrome and identify the oncogenes and tumor-suppressor genes that are involved in morphologic transformation and tumor progression. We investigated six cases of Richter’s syndrome in which both the CLL/SLL and the DLL components in each case were available for analysis. Using a variety of molecular genetic approaches we were able to show that Richter’s syndrome may occur through different pathways, ie, the DLL may be clonally related to or clonally distinct from the original CLL/SLL, and that specific oncogene or tumor-suppressor gene alterations

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do not appear to characterize the morphologic transformation and clinical progression of CLL/SLL to DLL.

MATERIALS AND METHODS

Patients and pathologic samples. Six cases of Richter's syndrome were included in this report based strictly upon the availability of sufficient quantities of fresh pathologic specimens of both the CLL/SLL and DLL components to perform the studies described below. Heparinized peripheral blood (PB) samples and lymph node biopsy specimens were collected during the course of routine clinical evaluation using standard diagnostic procedures and promptly delivered to the laboratory. Mononuclear cells were separated from the PB and lymph node samples by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. The mononuclear cells were cryopreserved in a viable state in fetal calf serum and dimethylsulfoxide at \(-170^\circ\text{C}\) until needed. Representative hematopoietic- and eosin-stained sections were prepared from portions of each lymph node biopsy that was routinely fixed in buffered formalin, B5, or Bouin's and embedded in paraffin. Representative portions of each tissue specimen were embedded in a cryopreservation solution (OCT compound, Miles, Elkhart, IN) and stored at \(-70^\circ\text{C}\). The diagnosis of Richter's syndrome was based upon correlative analysis of the clinical, morphologic, and immunophenotypic characteristics.

Immunophenotypic characterization. The immunophenotypic profiles of the CLL/SLLs and DLLs was determined at the time of diagnosis by immunohistochemical staining of frozen or paraffin tissue sections using a three step avidin-biotin immunoperoxidase technique or an immunoperoxidase phosphatase antialkaline phosphatase method, and/or by direct and indirect immunofluorescent flow cytomtery of isolated cells in suspension using the FACSscan fluorescent activated cell sorter (Becton Dickinson, Mountain View, CA) in conjunction with a large panel of polyclonal and monoclonal antibodies (MoAbs) as previously described.\(^{31}\)

DNA extraction. Genomic DNA was extracted from cryopreserved mononuclear cell suspensions and tissue blocks using a salting-out procedure.\(^{32}\) Briefly, the cells or frozen tissue sections were resuspended in 3 mL of nuclease lysis buffer containing 10 mmol TrisHCl, 400 mmol NaCl, and 2 mmol EDTA; 200 \(\mu\text{L}\) of 10% sodium dodecyl sulfate (SDS) and 500 \(\mu\text{L}\) of proteinase K solution (1 \(\mu\text{g}\) proteinase K in 1% SDS and 2 mmol EDTA) were subsequently added. After overnight digestion at 37°C, 1 mL of saturated NaCl was added. This mixture was centrifuged at 2,500 rpm for 20 minutes, and 2 volumes of ethanol were added to the supernatant to precipitate the DNA, which was washed several times in 70% ethanol.

Southern blot analysis. Five-microgram aliquots of genomic DNA were digested with the appropriate restriction endonucleases according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN), electrophoresed in 0.8 or 1% agarose gels, and transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH).\(^{33}\) The filters were hybridized in 50% formamide/3x standard-citrate (SSC) buffer at 37°C, washed at 0.2x SSC/0.5% SDS at 60°C for 2 hours and then autoradiographed at \(-70^\circ\text{C}\) for 16 to 48 hours as previously described.\(^{34}\)

DNA and synthetic oligonucleotide probes. The Ig heavy chain gene was investigated by hybridization of EcoRI and HindIII digested DNAs to an Ig heavy chain joining region probe (JH).\(^{35}\) The Ig light chain genes were studied by hybridization of EcoRI and BamHI digested DNAs to an Ig-\(\lambda\) light chain joining region probe (Jk)\(^{36}\) and an Ig-\(\lambda\) light chain constant region probe (Ca), respectively.\(^{37}\) The T-cell receptor \(\beta\) chain (T\(\beta\)) gene was investigated by hybridization of BamHI-digested DNAs to the T\(\beta\) gene constant region probe.\(^{38}\) The organization of the e-myloc locus was analyzed by hybridization of EcoRI- and HindIII-digested DNAs to the MCM13RC probe.\(^{39}\) The bel-1 locus of chromosome 11 was investigated by hybridization of HindIII and EcoRI digests of DNA to the 2.1-kb SacI fragment for the major translocation cluster (MTC) breakpoint and 460-bp PuI-I-SmlI genomic fragment designated p94PS that lies approximately 23.4-kb of the MTC.\(^{40}\) The bel-2 locus was investigated by hybridization of BamHI-digested DNAs to the pFL-1 and pFL-2 probes representing a portion of chromosome 18 at the major and minor bel-2 breakpoint regions, respectively.\(^{41,42}\) The Rbl1 locus was analyzed by hybridization of HindIII-digested DNAs using 5-Rb (0.7-kb, Hpa I-EcoRI) and 3-Rb (3.9-kb, EcoRI-EcoRI) subclones derived from the full-length Rbl1 cDNA (gift of S. Friend, Harvard Medical School, Charlestown, MA)\(^{43}\) corresponding to exons 1 to 9 and 10 to 27, respectively. The Epstein-Barr virus (EBV) genome was detected by hybridization of BamHI-digested DNAs to the 5.2-kb BamHI-EcoRI fragment of EBV genomic termini.\(^{44}\) Two highly polymorphic markers for loci on chromosome 17, pYNZ22.1\(^{45}\) and p144-DK\(^{46}\) were used to assess the loss of heterozygocity in the p53 mutated case, as well as to evaluate the possibility of p53 deletion in the nonmutated cases. The nitrocellulose filter of the EcoRI-digested DNAs of case number two, which was positive with JH, was rehybridized with case-specific CDR3 synthetic oligonucleotides (ACCCACCT TGGGTAACGGATACCCTCGGAGGGAGGTCAT and AGATCGGCCTGGTGTTGATCGTACG) at 5 antibiotics as previously described.\(^{31}\)

DNA amplification. Heavy-chain Ig gene rearrangement products were amplified using genomic DNA (1 \(\mu\text{g}\) in the presence of mixed oligonucleotide primers (ACACGGCGC/TGCGTGTTAT- TCTG) (FR3A) designed on consensus sequences for codons 89 through 95 (third framework region) of human Ig/variable region (Vh), and a universal Jh oligonucleotide primer (GGATGCGAACCTGAGGACCGTTA) (Jh-33). Oligonucleotides were synthesized by the solid-phase triester method. Both oligonucleotide primers are equipped with Cla I and EcoRI restriction sites, and a Kpn I and HindIII restriction site, respectively, enabling cloning and sequence determination of the PCR-amplified fragments. DNA was amplified in the presence of 220 \(\mu\text{mol}\) deoxynucleotides triphosphates (dNTPs), 50 mmol KCl, 10 mmol Tris-HCl pH 8.1, 1 mmol MgCl\(_2\), 1% gelatin, 20 pmol of each oligonucleotide and 2.5 U of Taq DNA polymerase. The mixture was overlaid with mineral oil and subjected to one cycle of 1 minute at 94°C, 3 minutes at 52°C, 40 minutes at 72°C, followed by 40 cycles of 1 minute at 94°C, 2 minutes, 30 seconds at 56°C, 90 seconds at 72°C, followed by a final extension of 7 minutes at 72°C performed on an automated heat block (DNA Thermal-Cycler, Perkin-Elmer Cetus). In all experiments, samples of genomic DNAs from mononuclear neoplastic B cells, normal reactive lymph nodes, tonsils, and T-cell leukemias were included as positive and negative controls. In addition, mixtures without template were included as a negative control. PCR products were size fractionated on alkaline 4% agarose gels by electrophoretic separation enabling the detection of PCR fragments of approximately 70 to 120 bp. Based on the fact that the tumor cell population comprised at least 10% of the total cells in all our neoplastic samples, discrete bands should be present if the neoplastic cells have undergone Ig gene rearrangement.

PCR-SSCP analysis. The sequences of p53 primers used for PCR amplification are as follows: P5-5, 5'-TTCCTTCTCCTG- CAGTACTC-3'; P5-3, 5'-AGTTGCACCGACGCTCAG-3'; P7-
The results of Southern blot analysis of the various restriction endonuclease-digested DNA samples for antigen-receptor gene rearrangements are depicted in Table 3 and illustrated in Figs 1 and 2. Clonal rearrangement of the Ig-heavy-chain gene was detected in all six pairs of DNA samples. The molecular size of the rearranged JH locus of the CLL/SLL and DLL components was identical in four cases and was different in two cases (Fig 1). Five of the six cases had more than one restriction-band fragment indicating rearrangement of both alleles. Analysis of the \( \kappa \) light chain gene in BamHI-digested DNAs showed clonal rearrangement in the CLL/SLL and DLL samples of four cases and in the CLL sample of one case (case 1). In the group of four cases where both samples were rearranged, the Southern blot patterns were identical in three cases and different in one case (case 2) (Fig 1, top). Clonal rearrangement of the \( \lambda \) light chain gene was detected in both the CLL and DLL samples in one case (case 6) and in the DLL sample of one case (case 1) (Fig 1, bottom). The 8-kb CA germline bands were missing because of restriction fragment length polymorphism in case 2.37 In summary, Southern blot analysis showed an identical Ig gene-rearrangement pattern in the CLL/SLL and DLL components in four cases and a different Ig gene-rearrangement pattern in the CLL/SLL and DLL components in two cases. The T-cell receptor \( \beta \) chain gene was present in the germline configuration in all the samples from all six patients (Table 3).

PCR amplification, cloning, and sequencing of the CDR3 region of the Ig-heavy chain gene. Cloning and sequencing studies were performed to determine whether differences in the Southern blot pattern of Ig gene rearrangement were the result of two different clones or somatic mutations. The two Richter’s syndrome cases where the CLL/SLL and the DLL components have different patterns of clonal Ig gene rearrangement, and one of the cases where they have identical patterns Clonal Ig gene rearrangement were selected for cloning and sequencing the CDR3 region of the Ig heavy chain gene. PCR amplification of the six samples from the three cases resulted in PCR products ranging in size between 120 and 75 bp. The physical maps and nucleotide sequences of the Ig-heavy chain CDR3 region isolated from the CLL/SLLs and DLLs of these three cases are illustrated in Fig 3. The framework segments (FR) 3 and 4 and CDR3 region are indicated according to Kabat et al.29 The CDR3 sequences of the SLL sample of case 1 showed two clonal pop-
The genetic defect of the parent cell is expressed in all of the members of the clone. However, the occasional biclonal derivation and histologic transformation of B-cell lymphomas indicates that additional genetic alterations of the neoplastic clone or the development of a new and different clone may occur. A minority of lymphomas that undergo transformation are low-grade follicular and diffuse small lymphocytic lymphomas. The morphologic transformation of CLL/SLL to DLL, which is usually associated with 63- and 42-bp long DNA sequences. The CDR3 region of the DLL sample from the same case consisted of 30 bp. Homologous CDR3 sequences were not detected in the SLL and DLL samples of case 1. Case 2 contained two clones in the CLL and two clones in the DLL that were identical. Somatic point mutations or deletions were not detected. The sequencing data of the CLL and DLL samples of case 3 showed identical clones.

**CDR3-specific oligonucleotide hybridization of the JH-probed Southern blot.** In case 2, the rehybridization of the JH-probed EcoRI-digested nitrocellulose filter with synthetic oligonucleotides specific for CDR3 showed a hybridization band at the site of the rearranged JH band in the CLL sample but not in the DLL sample (data not shown).

**EBV analysis.** The six DNA samples were digested with the BamHI restriction endonuclease and subjected to Southern blot hybridization using a DNA probe specific for the fused termini of the EBV genome. A lymphoid cell line derived by EBV infection of normal polyclonal B cells and an EBV⁺ clonal B-cell lymphoma line were used as controls for polymorphic and monomorphic EBV termini, respectively. The DLL sample from case 2 displayed a single intense positive band analogous to the monoclonal control (data not shown). None of the remaining CLL/SLL or DLL specimens contained evidence of EBV infection.

**Proto-oncogene and tumor-suppressor gene analysis.** The results of Southern blot and SSCP analysis of the c-myc, bcl-1, and bcl-2 proto-oncogenes and the Rb and p53 tumor-suppressor genes are presented in Table 4. Southern blot analysis showed the germline configuration of the bcl-1, bcl-2, c-myc, and Rb genes (not shown). PCR-SSCP analysis of the p53 gene showed three abnormally migrating bands: in exon 5 in the DLL sample of case 1, and in exon 1 in both the CLL and DLL samples of case 4 (Fig 4). The nature of the p53 gene mutations detected by SSCP analysis were further studied by cloning and sequencing of the PCR amplified exons. The DLL in case 1 had a G to T transition in the second nucleotide of codon 176 resulting in a change in the encoded amino acid from cysteine to phenylalanine (Fig 5). The DLL samples of case 4 contained the identical point mutation, a C to T transition in the first nucleotide of codon 282 leading to an alteration from arginine to tryptophane (Fig 3). Southern blot analysis of HindIII-digested DNA with 17 chromosome-specific pYNZ22.1 and p144-D6 probes showed only the mutated allele, implying loss of the normal allele (not shown).

**DISCUSSION**

Human B-cell lymphomas are generally believed to represent monoclonal proliferations of neoplastic B cells that originate from a single transformed lymphoid precursor. The genetic defect of the parent cell is expressed in all of the members of the clone. However, the occasional biclonal derivation and histologic transformation of B-cell lymphomas indicates that additional genetic alterations of the neoplastic clone or the development of a new and different clone may occur. The majority of lymphomas that undergo transformation are low-grade follicular and diffuse small lymphocytic lymphomas. The morphologic transformation of CLL/SLL to DLL, which is usually associated with).

### Table 2. Immunophenotype of Paired CLL/SLL and DLL Samples in Six Cases of Richter’s Syndrome

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<tr>
<th>Phenotypic Marker</th>
<th>% SLL</th>
<th>% DLL</th>
<th>% SLL</th>
<th>% DLL</th>
<th>% SLL</th>
<th>% DLL</th>
<th>% SLL</th>
<th>% DLL</th>
<th>% SLL</th>
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*Abbreviation: ND, not determined.*

### Table 3. Ig and T-Cell Receptor Gene-Rearrangement Analysis of Paired CLL/SLL and DLL Samples in Six Cases of Richter’s Syndrome

<table>
<thead>
<tr>
<th>cDNA Probes</th>
<th>Restriction Enzymes</th>
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<th>DLL</th>
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<td>R</td>
<td>≠</td>
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<td>R</td>
<td>G</td>
</tr>
<tr>
<td>J4</td>
<td>BamHI</td>
<td>R</td>
<td>G</td>
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*Abbreviations: G, germline configuration; R, gene rearrangement; =, gene rearrangement identical; ≠, gene rearrangement different.*
with clinical progression, has been designated Richter’s syndrome.
Two issues must be resolved in order to understand the
development of Richter’s syndrome: the clonal origin of
the DLL and the secondary biologic effects on the CLL/SLL
that are responsible for transformation.

There are two possible explanations for the occurrence of
two histologically different lymphomas in the same patient.
First, the neoplastic cell populations may represent two
different clones as a result of different transforming events.
Several reports describe cases of Richter’s syndrome in
which the CLL and the DLL express different Ig iso-
types. In addition, Southern blot analysis of Ig gene
rearrangements has not shown concordant rearrangement
bands in the CLL and DLL components of individual cases
of Richter’s syndrome. According to these reports, Rich-
ter’s syndrome may be biclonal. However, in the transfor-
mation of follicular lymphoma the altered DNA sequences
are the result of somatic mutations. Because somatic mu-
tations change restriction-enzyme sites, the discordant
Southern blot analyses do not necessarily indicate unre-
lated clonal development. In the present study, Southern
blot analyses of Ig gene rearrangements showed discordant
bands in two cases (cases 1 and 2). We performed PCR am-
plication, cloning, and sequencing of the CDR3 region
of the Ig-heavy chain gene to determine whether somatic
mutations or the development of a new clone is responsible
for these different Ig gene-rearrangement patterns on the
Southern blot. Two different CDR3 sequences were found
in the SLL sample from case 1 indicating rearrangement of
both alleles. Because the CDR3 sequence of the DLL sam-
ple from case 1 did not match any of the SLL sequences,
case 1 represents a case of Richter’s syndrome where the
DLL is unrelated to the original SLL. In contrast, despite
the different Ig gene-rearrangement patterns by Southern
blot analysis in case 2, the CDR3 sequences of the CLL and
DLL components were identical. The discordant data of
sequence and Southern blot analysis can be interpreted in
different ways. One, the two neoplasms represent differ-
en clones and JH33 and/or FR3 primers do not recognize
the Ig sequence of the DLL, but do amplify the CLL. Al-
ternatively, the DLL developed from the CLL, and represent
the same clones, and somatic mutations that modified the
restriction sites lie outside of the sequenced CDR3 regions.
To exclude one or the other possibility, the JH-probed ni-
trocellulose filter was rehybridized with case-specific
CDR3 synthetic oligonucleotides. Because only the CLL
dNA sample showed hybridization with the CDR3 probes,
the nonidentical JH-rearranged band of the DLL sample
represents a new clone that was not amplified by the FR4-
JH33 PCR. However, further data concerning the clonal
relationship in this case is provided by the results of EBV-
DNA analysis. The integration of EBV genome in the DLL
may occur before or after the morphologic transformation
of CLL. If the infection is a posttransformation event, the
integrated EBV termini should show a polymorphic pat-
ttern on Southern blotting. If EBV infected the parent cell
before expansion of the tumor cell population, the South-
ern blot will show a monomorphic pattern. Southern blot
analysis showed a monomorphic pattern in this case, sug-
gest that this clonal expansion derived from a progeni-
tor B cell which was already infected by EBV. Meanwhile,
the DLL sample from case 2 was EBV-. Therefore, the CLL
and DLL cells do not apparently represent the same clone
in this instance.

The other model for the development of Richter’s syn-
drome proposes that the two neoplasms are identical B-cell
clones, and that other factors are responsible for the transformation without changing the B-cell clone. Reports documenting the same Ig isotype and anti-idiotypic expression,\textsuperscript{4,8.5} identical chromosome aberrations,\textsuperscript{6,14} similar reactivity with MoAbs,\textsuperscript{20} and matching Ig gene-rearrangement patterns\textsuperscript{9,13} support this theory. In our report, four of the six cases exhibited identical Ig gene-rearrangement patterns by Southern blot analysis, and DNA sequence analysis of the CLL/SLL and DLL of one case showed identical clones. In the multistep process of tumorigenesis, Nakamine et al\textsuperscript{55} suggested, according to their Southern blot data, that a series of somatic point mutations alter the CLL/SLL cells to develop DLL. Cherepakhin et al\textsuperscript{57} reported a case where the Ig VH sequences of the CLL and DLL differed in several somatic point mutations. Because the precise sites of somatic mutations of Cherepakhin’s case are not published, it is not clear whether the antigen-driven somatic mutation of CDR regions of the VH gene play a role in the development of Richter’s syndrome.\textsuperscript{58}

The CDR3 region including variable (V), diversity (D), and joining (J) segments, is the most unique region of the Ig gene.\textsuperscript{59} The occurrence of somatic point mutations is highest in the CDR3 region because of the random assortment of V, D, and J segments and imprecise joining mechanisms.\textsuperscript{60} Though somatic point mutations of the CDR3 region were not detected in our cases, other segments could be mutated. Based on sequencing data of CLL cases, the frequency and sites of somatic mutations are not obvious. Lack of somatic mutations,\textsuperscript{61} random distribution of somatic mutations,\textsuperscript{62,63} and selective CDR1 mutation of the VH region\textsuperscript{64} have been reported as well. The molecular genetic analysis of our six cases of Richter’s syndrome in conjunction with other analyses of individual cases indicates that the histologic transformation of CLL to DLL does not occur in a single homogeneous manner. Apparently, the DLL in Richter’s syndrome may represent either a clonal progression of the original CLL or a second lymphoid malignancy.

We investigated the involvement of several proto-oncogenes and tumor-suppressor genes in the progression of CLL to DLL using Southern blot and SSCP analysis. We studied loci that are known to be involved in B-cell malignancies,\textsuperscript{28,29,65-67} and are suspected of playing a role in lymphoma progression.\textsuperscript{25,26,59} The examination of large numbers of CLls has shown no or only rare cases containing bcl-1, bcl-2, or c-myc rearrangement.\textsuperscript{25,26,66} Some DLLs have been found to be bcl-1 or bcl-2 rearranged,\textsuperscript{29} and Ince

\begin{table}
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\begin{tabular}{|l|l|l|l|l|l|}
\hline
\textbf{1. DLL-1} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{1. DLL-2} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{1. DLL-3} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{1. DLL-4} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{1. DLL-5} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\hline
\textbf{2. CLL-1} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{2. CLL-2} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{2. CLL-3} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{2. CLL-4} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{2. CLL-5} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\hline
\textbf{3. CLL-5} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{3. CLL-10} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{3. CLL-11} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{3. DLL-2} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{3. DLL-3} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{3. DLL-4} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{3. DLL-5} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\textbf{3. DLL-9} & AAGGGCCTTATTATTAGGCTGCTAAGCG & TGGGGCAAGGAAAGGGTGACCTCAAGCTGCTAAGCG & \\
\hline
\end{tabular}
\caption{Clonal nucleotide sequences of FR3A-JH33 PCR product of paired CLL/SLL and DLL samples in three cases of Richter’s syndrome. The FR3, CDRB, and FR4 segments are indicated according to Kabat et al\textsuperscript{80} and are demarcated with solid horizontal lines. Different sequences are indicated by appropriate base changes.}
\end{table}
et al suggested that bcl-1 rearranged DLLs represent cases of transformed CLL/SLL. By contrast, other studies, including our own, have not found the bcl-1 locus rearranged in DLLs. The possible effect of the c-myc oncogene on tumor progression is described in a case of blast transformation of follicular lymphoma, and clinically aggressive CLL, but increased c-myc oncogene expression has been detected in blastic transformation of CLL without gene rearrangement.

It has been proposed that inactivation of the Rb locus may contribute to the progression of human malignancies. Using Southern blot analysis, as many as 13% of CLLs have been shown to exhibit Rb gene deletion. Ginsberg et al described Rb mutations in low-grade lymphomas with a poor clinical prognosis and suggested that mutations in the Rb locus contribute to progression of the lymphoid neoplasm. However, in our study, neither the CLL/SLL nor the DLL components of the six cases of Richter’s syndrome showed Rb gene deletion. Oscier et al reported a case of Richter’s syndrome where the CLL cells but not the DLL cells were mutated, also suggesting that Rb gene deletions do not contribute to histologic transformation of CLL. Recently, however, using the more sensitive fluorescent in situ hybridization (FISH) technique, as many as 31% of CLLs have been shown to contain Rb gene deletion. Therefore, application of the FISH technique, which is more sensitive than Southern blotting, in the future may better help to delineate the role of Rb gene deletion in Richter’s syndrome.

The p53 gene is one of the tumor-suppressor genes that may be involved in negative regulation of cell proliferation. Several lines of evidence support the notion that loss, alteration, or inactivation of this gene contributes to the development and progression of human malignancies. Gaidano et al investigated the frequency of p53 mutations in a large series of B-cell malignancies and found histologically transformed cases of CLL (Richter’s syndrome) to be frequently mutated. Ichikawa et al described p53 gene mutations in a large series of clinically advanced B-cell lymphomas. However, neither of the two studies analyzed the tumor cell populations from the same patients both before and after transformation. We performed SSCP and sequence analysis of exons 5 through 9 of the p53 gene in six paired

### Table 4. EBV, Proto-oncogene, and Suppressor Gene Analysis of Paired CLL/SLL and DLL Samples in Six Cases of Richter’s Syndrome

<table>
<thead>
<tr>
<th>cDNA Probes and Primers</th>
<th>restriction Enzymes</th>
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<tr>
<td>EBV-TR</td>
<td>BamH I</td>
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<td>bcl-1 (MTC)</td>
<td>Hind III</td>
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<td>bcl-1 (p94PS)</td>
<td>EcoR I</td>
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<td>bcl-2 (pFL-1)</td>
<td>BamH I</td>
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<td>bcl-2 (pFL-2)</td>
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Abbreviations: G, germ line; M, mutated; WT, wild type; N, negative; P, positive.

Fig 4. PCR-SSCP analysis of p53 gene mutations in six cases of CLL/SLL and the subsequent DLL. Representative examples are shown for exons 5 and 8. The lane at the left end (U) indicates undenatured control DNA. The arrows show aberrantly migrating fragments.
samples of CLL and the subsequent DLL in order to determine the possible role of the p53 gene in tumor progression and transformation. We detected mutations in exon 5 (codon 176) of the DLL in case 1 and in exon 8 (codon 282) of both the CLL and the DLL in case 4. The exon 8 mutations in the CLL and DLL were identical. We did not detect p53 gene mutations in the other four cases of Richter’s syndrome. It is obvious that p53 mutation was not primarily responsible for morphologic transformation in case 4, because the identical p53 mutation was present both before and after histologic transformation in both the CLL and DLL. In case 1, the CLL and DLL clones were different and only the DLL cells were p53 mutated. Therefore, the p53 mutation may have played a role in the development of a new clone rather than in the morphologic transformation of the CLL/SLL in this case.

This study addressed the issue of the clonal relationship between CLL and the subsequent DLL in Richter’s syndrome, and sought to identify the factors responsible for transformation. We showed that the DLL may represent either the identical clone as the CLL/SLL, or may represent a new unrelated clone, and that none of the well-known oncogenes and tumor-suppressor genes are responsible for the transformation of CLL/SLL to DLL. Because the DLL may develop from the original CLL/SLL or may represent a different malignant clone, the transforming events involved in the development of Richter’s syndrome may be different as well. In those cases of Richter’s syndrome where the DLL represent the same clones, random or antigen-driven somatic mutations may be involved in the progression of the lymphoma. In the development of biclonal Richter’s syndrome, a genetic malformation that has no effect on the CLL cells, such as p53 gene mutation, may initiate the second malignancy.

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Molecular genetic demonstration of the diverse evolution of Richter's syndrome (chronic lymphocytic leukemia and subsequent large cell lymphoma)

A Matolcsy, G Inghirami and DM Knowles