

# Analysis of Alterations of Oncogenes and Tumor Suppressor Genes in Chronic Lymphocytic Leukemia

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***B cell chronic lymphocytic leukemia (B-CLL) represents the most frequent adult leukemia in the Western world. The molecular pathogenesis of B-CLL is largely unknown. Although initial reports on small panels of cases had suggested a role for Bcl-1 and Bcl-2 oncogene activation in B-CLL, later investigations failed to confirm these data. Among tumor suppressor genes, p53 mutations have been reported in a fraction of cases. In this study, we have attempted a conclusive definition of the involvement of dominantly acting oncogenes (Bcl-1 and Bcl-2) and tumor suppressor loci (p53, 6q<sup>-</sup>) in 100 cases of B-CLL selected for their CD5 positivity and Rai's stage (0 to IV). Rearrangements of Bcl-1 and Bcl-2 and deletions of 6q and 17p were analyzed by Southern blot using multiple probes. Mutational analysis (single strand conformation polymorphism and polymerase chain reaction direct sequencing) was used to assay p53 inactivation. No alterations of Bcl-1 or Bcl-2 were detected in the 100 cases tested. Mutations of p53 were found in 10/100 cases without any significant association with clinical stage. Deletions of 6q were present in 4/100 cases. Overall, our data indicate that: 1) contrary to previous reports, Bcl-1 and Bcl-2 rearrangements are not involved in CD5<sup>+</sup> B-CLL pathogenesis and 2) p53 mutations are present in 10% of cases at all stages of the disease. (Am J Pathol 1994, 144:1312-1319)***

B cell chronic lymphocytic leukemia (B-CLL) is a malignancy of CD5<sup>+</sup> B lymphocytes, a subpopulation of B cells present at low levels in the normal adult. B-CLL represents the most frequent type of all adult leukemias in the Western world.<sup>1,2</sup> Indeed, in some geographical areas, B-CLL accounts for up to 40% of leukemias arising in the adult over 40 years of age.<sup>1,2</sup> The clinical course of B-CLL is highly variable, including very indolent and rapid, aggressive forms that may represent late stages of the disease or the transformation of B-CLL into Richter's syndrome. The most commonly used indicators of prognosis are the extent and clinical features of the disease, classified according to Rai's staging system, together with the kinetics of proliferation of the neoplastic B lymphocyte population.<sup>1-4</sup> Other factors, ie, karyotypic abnormalities and immunophenotypic features, may also have an independent prognostic value.<sup>1,2,5-7</sup>

The molecular pathogenesis of B-CLL is largely unknown. Cytogenetic observations have revealed a number of recurring chromosomal abnormalities, including trisomy 12 and deletions involving band 13q14, the site of the RB1 gene, although the relevant genes have not been identified.<sup>1,2,5,6</sup> The involvement of dominantly acting oncogenes known to play a role in B cell neoplasia, such as *Bcl-1* and *Bcl-2*, is controversial.<sup>1,2,8,9</sup> Although initial studies suggested a significant role for *Bcl-1* and *Bcl-2* in B-CLL pathogenesis,<sup>10-16</sup> their actual involvement has been questioned by later investigations.<sup>17-20</sup> Alterations of other dominantly acting oncogenes, such as *c-myc* and the *ras* family genes, have never been found in

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B-CLL.<sup>17,18,21</sup> Among tumor suppressor genes, we and others have reported the presence of p53 inactivation in a subset of B-CLL.<sup>22-24</sup> The frequency of this lesion appears to significantly increase on B-CLL transformation into the aggressive lymphoma-like disease known as Richter's syndrome.<sup>22</sup> Whether p53 mutations have any prognostic value in the course of B-CLL and/or cluster with the advanced stages of B-CLL (ie, Rai's stages III and IV) remains an open question.

In this study we have analyzed a panel of 100 cases of B-CLL representing different stages of the disease for a number of genetic lesions, including rearrangements of the oncogenes *Bcl-1* and *Bcl-2*, inactivation of the tumor suppressor gene p53, and deletions of 6q. The scope of the study was to conclusively define the frequency of each genetic lesion in a large number of cases representing all stages of disease and to investigate the associations between genetic lesions and the clinical behavior of B-CLL.

## Materials and Methods

### Patients

Samples of peripheral blood (PB) were collected during standard diagnostic procedures from 100 B-CLL patients referred to the Hematology Service, Institute of Medical Sciences, University of Milan, Italy. Diagnosis of B-CLL was according to the criteria of the International Workshop on Chronic Lymphocytic Leukemia and the National Cancer Institute-sponsored Working Group, which included sustained lymphocytosis of greater than  $10 \times 10^9/L$  in PB with mature appearing lymphocytes and bone marrow (BM) lymphocytosis of at least 30% in BM aspirates.<sup>25,26</sup> The disease was staged according to Rai's staging system.<sup>3</sup> Lymphocyte doubling time (LDT) was calculated as previously reported.<sup>4</sup> In all patients, the neoplastic lymphocytes co-expressed CD19 and CD5, as demonstrated by immunophenotypic analysis of cell surface markers. Following the recommendation of the Fifth International Workshop on CLL, which restricts the definition of B-CLL to cases expressing CD5 (27), no cases of CD5-negative B-CLL were included in this study. Moreover, all the cases were CD23 positive. The presence of B cell derived monoclonal populations was demonstrated for all patients based on immunophenotypic analysis of cell surface immunoglobulins (SmIg) and immunogenotypic analysis of immunoglobulin gene rearrangements. For most cases, the fraction of malignant cells, iden-

tified by their CD19/CD5 expression, in the pathological specimen was greater than 80% and in all cases greater than 45%, as demonstrated by immunophenotypic analysis. For all cases a mononuclear cell suspension of more than 95% viability was prepared by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation.

### DNA Extraction and Southern Blot Analysis

Genomic DNA was prepared from mononuclear cell suspensions by cell lysis, proteinase K digestion, "salting out" extraction, and ethanol precipitation as described.<sup>28</sup> For Southern blot analysis,<sup>29</sup> 6  $\mu$ g of DNA was digested with the appropriate restriction endonuclease, electrophoresed in 0.8% agarose gel, denatured, neutralized, transferred to Duralose filters (Stratagene, La Jolla, CA), and hybridized to probes that had been labeled with <sup>32</sup>P by random primer extension.<sup>30</sup> Filters were washed in 0.2 $\times$  standard saline citrate (SSC) (1 $\times$  SSC = 0.15 M NaCl + 0.015 M sodium citrate)/0.5% sodium dodecyl sulfate (SDS) for 2 hours at 60 C, and then autoradiographed using intensifying screens (Quanta III, Dupont).

### DNA Probes

Immunoglobulin (Ig) gene rearrangement analysis was performed using a J<sub>H</sub> probe (a gift of Dr. S. Korsmeyer)<sup>31</sup> on *Hind*III, *Eco*RI, and *Bam*HI digests. The configuration of the *Bcl-2* locus was investigated using probes pFL-1 and pFL-2 (a gift of Dr. M. L. Cleary)<sup>32</sup> on *Bam*HI digests and probe pB16 (a gift of Dr. Y. Tsujimoto)<sup>33</sup> on *Hind*III digests. The *Bcl-1* locus was studied using probe MTC (probe a and probe b a gift of Dr. Y. Tsujimoto)<sup>34</sup> and probe p94PS (a gift of Dr. T. C. Meeker)<sup>35</sup> on *Bcl*I, *Hind*III, and *Eco*RI digests. Loss of heterozygosity at 17p13, the site of the p53 locus, was studied using the two highly polymorphic probes pYNZ22.1/D17S5<sup>36</sup> and p144-D6/D17S34<sup>37</sup> on *Hinf*I digests, as previously reported.<sup>38</sup> Amplification of the MDM2 gene was tested using the c14-2 probe (kind gift of Dr. B. Vogelstein) on *Eco*RI digests as described.<sup>39</sup> The presence of 6q deletions was studied using two highly polymorphic cosmid clones mapping to 6q27: CEB3/D6S132 and CEB4/D6S133 (a gift of Dr. G. Vergnaud)<sup>40</sup> on *Hae*III and *Pvu*II digests, respectively.

### Oligonucleotides, Single Strand Conformation Polymorphism (SSCP) Analysis, and Direct Sequencing of Polymerase Chain Reactions (PCR) Products

The oligonucleotides used to amplify sequences of the p53 gene exons 5 through 9 have been previously reported.<sup>22</sup> SSCP analysis was modified from Orita et al<sup>41</sup> and performed as previously described.<sup>22</sup> Sequencing reactions were performed as previously reported.<sup>22</sup>

## Results

### Patient Characteristics

The panel of 100 B-CLL cases included in this study was selected for being representative of the various clinical features of the disease. Patients were representative of the different stages according to Rai (stages 0 to IV): 18 cases were analyzed while in Rai's stage 0, 35 in stage I, 38 in stage II, and 9 in stages III and IV. The proliferative kinetics of the disease was also taken into account and scored as LDT, which represents an independent prognostic marker. LDT was >36 months in 36 cases, 12 to 36 months in 19 cases, and <12 months in 14 cases; in the other cases LDT could not be evaluated and was indicated as 0. Finally, for each Rai's stage and LDT value, the patients included were representative of different therapeutic statuses.

Overall, 49 cases had received treatment with chlorambucil and prednisone, including 6/18 cases in stage 0, 14/35 in stage I, 21/38 in stage II, and 8/9 in stages III and IV, whereas the other 51 cases were studied before onset of therapy. With respect to LDT, 9/12 cases with LDT <12 months, 14/19 with LDT 12 to 36 months, and 10/36 with LDT >36 months had received chemotherapy. In all cases tested, a monoclonal pattern of Ig gene rearrangement was observed by Southern blot analysis using a J<sub>H</sub> probe on EcoRI, HindIII, or BamHI digestions (Figure 1). The Smlg were weakly expressed in 98 cases (IgM/D class in 69 cases, IgM alone in 24, and IgD alone in 5) and undetectable in 2 cases. In all patients, the neoplastic lymphocytes co-expressed CD19 and CD5 and were CD23 positive. Therefore, only "true" B-CLL, as defined by the Fifth International Workshop on CLL,<sup>27</sup> were included in this study.

### BCL-1 and BCL-2 Studies

Chromosomal translocations involving chromosome 11q13, in the case of *Bcl-1*, or chromosome 18q21, in the case of *Bcl-2*, and an antigen receptor locus result in rearrangements of the *Bcl-1* and *Bcl-2* loci observed in B cell neoplasia.<sup>8,9</sup> In the case of *Bcl-2*, at least three breakpoint cluster regions have been identified that can be explored by Southern blot analysis using different DNA probes.<sup>32,33</sup> In the case of *Bcl-1*, most cases rearrange within the major translocation cluster (MTC) region of the locus, although sporadic breakpoints have also been found outside

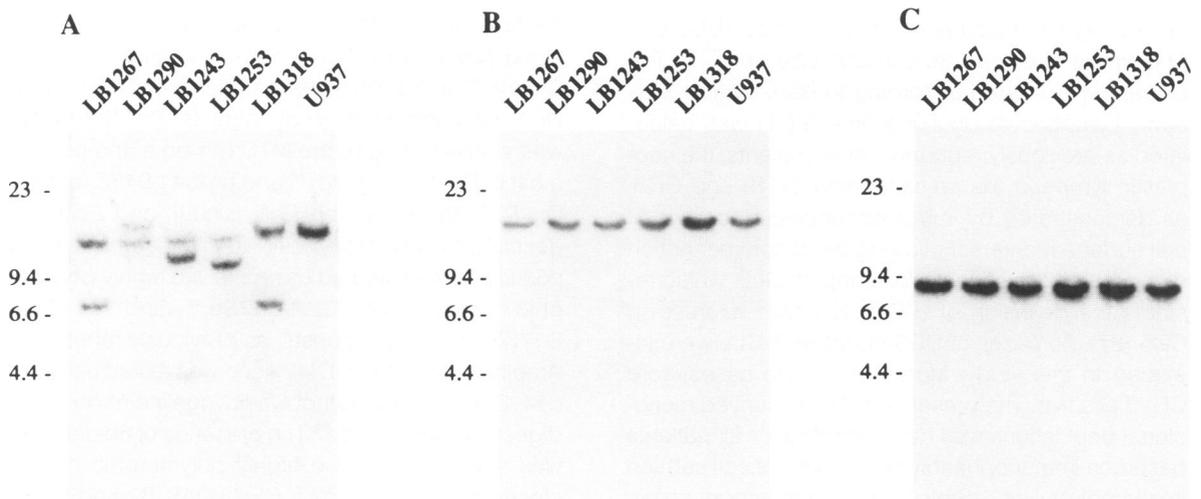


Figure 1. Immunogenotypic analysis (A), and molecular characterization of *Bcl-1* (B) and *Bcl-2* (C) in B-CLL. Genomic DNAs were digested with HindIII (A and C) or Bcl-1 (B) and subjected to Southern hybridization using probes representative of J<sub>H</sub> (A), the *Bcl-1* locus (probe a, B), and the *Bcl-2* oncogene (probe pB16, C). U937 represents a germ line control for the Ig genes. All B-CLL cases, indicated by the letters LB followed by a serial number, displayed a clonal rearrangement of the Ig genes. In all cases, *Bcl-1* and *Bcl-2* exhibited a germ line pattern.

**Table 1.** Frequency of Genetic Lesions in B-CLL

	<i>Bcl-1</i> *	<i>Bcl-2</i> *	p53*	6q*
Stage 0	0/18	0/18	2/18	0/18
Stage I	0/35	0/35	2/35	3/35
Stage II	0/38	0/38	4/38	1/38
Stages III and IV	0/9	0/9	2/9	0/9

\* Positive/tested.

this cluster.<sup>34,35</sup> In this study, 100 cases of B-CLL were subjected to Southern blot analysis using three different probes each for the *Bcl-2* and *Bcl-1* loci on multiple restriction endonuclease digests (Figure 1). The *Bcl-2* probes used explored all three known translocation clusters, including the major cluster region (MCR), the minor breakpoint region (MBR), and the cluster region located in the 5' portion of the gene.<sup>32,33</sup> With respect to *Bcl-1*, the MTC was explored with two probes, whereas a third probe tested a breakpoint site located 5' of the MTC.<sup>34,35</sup> No rearrangements of *Bcl-1* or *Bcl-2* were found in any of the cases tested (Table 1).

### The p53 Gene Inactivation

Inactivation of the p53 tumor suppressor gene in human tumors is most frequently caused by point mutations in the coding sequence of exons 5 through 9 in one p53 allele with or without loss of the other wild-type allele.<sup>42,43</sup> In rare instances, mainly represented by sarcomas, p53 inactivation occurs as a result of amplification of the MDM2 gene, the product of which inactivates p53 by binding to it.<sup>39</sup> Both mechanisms of p53 inactivation were assayed in the B-CLL cases included in this study.

First, the occurrence of p53 mutations in B-CLL was tested by a two-step strategy, as previously reported.<sup>22</sup> Exons 5 through 9 of the p53 gene were analyzed in genomic DNA of 100 B-CLL cases by the PCR-SSCP technique (Figure 2). Fragments displaying an altered electrophoretic mobility by SSCP

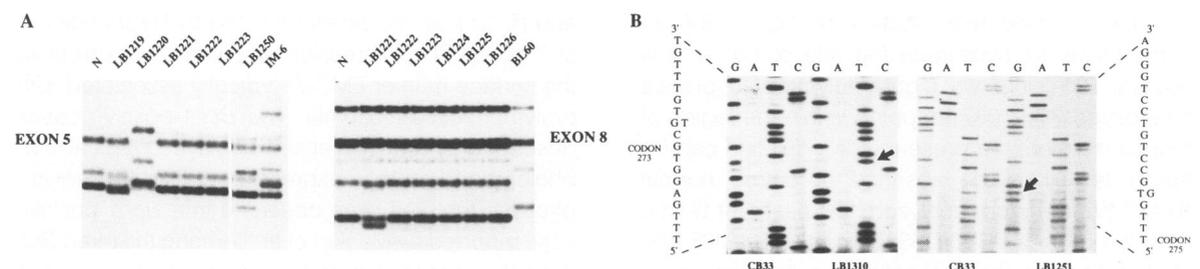
analysis were then reamplified in a separate reaction and analyzed by PCR direct sequencing to confirm and characterize the mutation (Figure 2). Second, MDM2 amplification was tested by Southern blot hybridization.<sup>39</sup>

The p53 mutations were detected in 10% of B-CLL cases (10/100; Table 1), whereas no patient showed amplification of the MDM2 gene (data not shown). Overall, p53 mutations were detected at all stages of the disease, including 2/18 patients at stage 0, 2/35 at stage I, 4/38 at stage II, and 2/9 at stages III-IV. In addition, mutations were detected both before chemotherapy (2 cases) and after it (8 cases). LDT was 12 to 36 months in 3 of the patients harboring p53 mutations, <12 months in 1 case, and 0 in 6 cases. The clinical features of the patients harboring p53 mutations, together with the characteristics of the p53 mutation, are described in Table 2. Overall, the type of lesion of p53 in B-CLL are similar to those found in other lymphoid malignancies.<sup>22,43</sup> The majority of mutations (10/11 events) were represented by single base pair substitutions, leading to a missense (9 cases) or a nonsense (1 case) mutation. One sample displayed a point deletion.

In the majority of human tumors, p53 mutations are accompanied by the deletion of the normal allele at 17p13, the site of the p53 locus.<sup>42,43</sup> Therefore, we tested the presence of loss of heterozygosity (LOH) at 17p13 in B-CLL using two highly polymorphic probes mapping to this region as previously reported.<sup>38,44</sup> LOH for 17p13 was detected in 6 of the 10 cases with a p53 gene mutation (Figure 3, Table 2).

### Other Tumor Suppressor Loci

Recent molecular analysis of 6q<sup>-</sup> in B cell neoplasia has shown that 6q deletions are a relatively frequent event in some subsets of B cell malignancies, ie, B-NHL, and that molecular analysis allows the de-



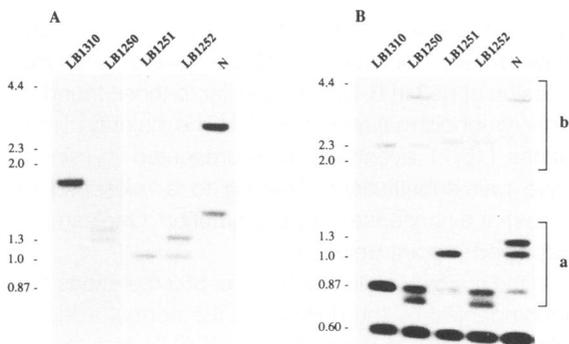
**Figure 2.** Analysis by PCR-SSCP (A) and PCR direct sequencing (B) of the p53 gene in B-CLL. A: PCR-SSCP: p53 exons 5 and 8 are shown. Samples were scored as positive when their migration pattern differed from the normal control (N). IM-6 and BL60 represent positive controls for exons 5 and 8, respectively (ref. 22). B: PCR direct sequencing: genomic sequence analysis was performed for samples scored as positive by the PCR-SSCP assay to confirm and characterize the mutation. Cases LB1310 and LB1251 are shown. CB33 represents a lymphoblastoid cell line used as wild-type control. The coding strands are shown for both cases. Mutations are indicated by arrows; the codon at which the mutation occurs is also shown.

**Table 2.** Summary of p53 Mutations in B-CLL

Patient	Stage*	LDT†	Codon	Mutation	Amino Acid Substitution	17p13 LOH
LB1250	0	<12	179	CAT→CTT	Hys→Leu	No
			273	CGT→GGT	Arg→Gly	
LB1310	0	0	273	CGT→CTT	Arg→Leu	Yes
LB1304	I	12-36	234	TAC→TGC	Tyr→Cys	Yes
LB1261	I	12-36	154	GGC→AGC	Gly→Ser	No
LB1221	II	12-36	283	CGC→TGC	Arg→Cys	Yes
LB1219	II	0	136	CAA→CAΔ	Frameshift	No
LB1303	II	0	175	CGC→CAC	Arg→Hys	No
LB1220	II	0	165	CAG→TAG	Gln→Stop	Yes
LB1251	III-IV	0	275	TGT→TGG	Cys→Trp	Yes
LB1273	III-IV	0	175	CGC→CAC	Arg→Hys	Yes

\* Patients were staged according to Rai's staging system (ref. 3).

† LDT (ref.4). LDT was scored as follows: <12 months; 12 to 36 months; >36 months; 0, not evaluable.



**Figure 3.** LOH analysis at 17p13 in representative B-CLL cases. Genomic DNAs were digested with *Hin*I subjected to Southern blot hybridization and probed with the highly polymorphic clones pYNZ22.1 (A) and p144-D6 (B), mapping to the vicinity of p53. pYNZ22.1 recognizes one allelic system. The p144-D6 recognizes two allelic systems (a and b, identified by brackets on the side of the figure) and two constant bands, one of which co-migrates with one of the alleles of system a. Cases were scored as positive for LOH when displaying only one allele in all three allelic systems, as previously reported (refs. 38, 44). Among the cases shown, LB1310 and LB1251 display only one allelic band with both probes, indicating the presence of LOH. On the contrary, all the other cases, including the normal control (N) show two bands in all three allelic systems. In the case of LB1310, LB1250, and LB1252 one allele of the allelic system a recognized by p144-D6 co-migrates with a constant band.

tection of small 6q deletions undetectable at the cytogenetic level.<sup>45</sup> These observations, coupled with the lack of molecular studies of 6q in B-CLL, prompted us to investigate the role of 6q<sup>-</sup> in this disease. By using two highly polymorphic probes (heterozygosity >90%) mapping within the region of minimal deletion, the presence of a deletion can be rapidly tested in the absence of paired normal DNA.<sup>46</sup> We therefore analyzed 100 cases of B-CLL using the probes CEB3/D6S132 and CEB4/D6S133, which recognize the presence of a deletion undetectable at the cytogenetic level. A 6q deletion was scored in 4/100 cases (Table 1), including 3 cases at stage I and 1 case at stage II. LDT was >36 months in 2 cases, <12 months in 1 case, and 0 in 1 case. All

cases harboring a 6q deletion displayed only wild-type p53 sequences.

### Discussion

In this study we have performed a comprehensive molecular analysis of the *Bcl-1*, *Bcl-2*, and p53 genes and 6q deletions in 100 cases of B-CLL patients at different stages of the disease. Following the recommendations on the Fifth International Workshop on CLL regarding the definition of "true" B-CLL,<sup>27</sup> only CD5<sup>+</sup> CD23<sup>+</sup> cases were included in this study. We aimed to conclusively define the frequency of each genetic lesion in a large panel of cases and testing the association between the presence of a genetic lesions and the clinical behavior of the tumor.

No alterations of *Bcl-1* and *Bcl-2* were detected in our B-CLL panel. In this respect, our results are at variance with previous studies that had detected rearrangements of these genes in a variable proportion of cases.<sup>10-20</sup> However, most *Bcl-1*- and/or *Bcl-2*-positive cases reported in the literature displayed peculiar phenotypic features inconsistent with a diagnosis of true B-CLL, according to the Fifth International Workshop on CLL. Indeed, the *Bcl-1*- and *Bcl-2*-positive cases reported by Raghoebier et al<sup>18</sup> lacked CD5 expression, whereas they expressed the surface marker FMC-7, typically associated with prolymphocytic leukemia. The *Bcl-1*-positive cases described by Newman et al<sup>20</sup> displayed atypical morphology and surface expression of CD11b. Prolymphocytic features were observed in a *Bcl-1*-positive case reported by Meeker et al<sup>16</sup> Among the three *Bcl-2*-positive cases reported by Adachi et al,<sup>12</sup> only two were known to express CD5 and, moreover, the expression of CD23 was not assessed in any of the cases. Finally, the original B-CLL case from which *Bcl-1* had been cloned was rediagnosed as centro-

cytic lymphoma at a later examination of the sample.<sup>47</sup> Thus, our comprehensive analysis of *Bcl-1* and *Bcl-2* in 100 cases of CD5<sup>+</sup> CD23<sup>+</sup> B-CLL rules out that *Bcl-1* and *Bcl-2* rearrangements play a role in the pathogenesis of true B-CLL, and suggests that cases originally found to harbor *Bcl-1* and *Bcl-2* lesions may represent other lymphoproliferative disorders biologically distinct from B-CLL.

Deletions of 6q have been regarded as a frequent event in B cell neoplasia and it has been recently shown that molecular assays are superior to conventional cytogenetics in detecting these abnormalities.<sup>45</sup> Here we have tested the molecular occurrence of 6q<sup>-</sup> in B-CLL. Our data indicate that 6q<sup>-</sup> are a rare event in B-CLL when compared with other B cell malignancies such as B cell non-Hodgkin lymphoma or acute lymphoblastic leukemia.<sup>9</sup> A similar frequency of 6q abnormalities in B-CLL was also detected in a previous cytogenetic study.<sup>48</sup>

Among the genetic lesions analyzed in this study, p53 represents the only gene found to be involved in a significant proportion of cases (10%) of B-CLL. Inactivation of p53 is found at all stages of the disease, suggesting that it may represent an early lesion at least in some cases. Because p53 mutations are also present in 50% of cases of Richter's syndrome,<sup>22</sup> it remains to be established whether B-CLL patients harboring p53 mutations are more prone to develop a Richter's syndrome or, conversely, whether p53 mutations occur *de novo* during Richter's transformation. In the case of tumor progression of other lymphoid malignancies, ie, transformation of follicular lymphoma to diffuse lymphoma, p53 mutations are thought to occur at the time of histological evolution.<sup>49,50</sup> In the case of B-CLL, studies with sequential samples of B-CLL and Richter's syndrome are required to clarify this issue. The mutational spectrum of p53 mutations in B-CLL resembles the one reported in the majority of human tumors, including other types of B cell neoplasia.<sup>22,42,43</sup> The frequent occurrence of p53 mutations at CpG dinucleotides, reported for most human tumors and confirmed here also in the case of B-CLL, suggests that these mutations occur spontaneously as DNA replication errors in the majority of cases.<sup>43</sup>

Based on the data presented here, it is clear that p53 inactivation may only partially account for the molecular pathogenesis of B-CLL. Trisomy 12 and deletions of 13q14, the most common cytogenetic abnormalities in B-CLL,<sup>5,6</sup> are thought to harbor oncogenes or tumor suppressor genes relevant for B-CLL development. The frequency of these two lesions in B-CLL, together with their clinical usefulness

as prognostic indicators, warrant a systematic search of the genes involved.

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