We studied 53 patients with B-cell chronic lymphocytic leukemia (B-CLL) and found mutations of the p53 gene in 15%. Patients with p53 gene mutations were found to have an aggressive form of B-CLL disease characterized by advanced Rai stage, rapid lymphocyte doubling time (LDT), and resistance to chemotherapy. While 27 of 29 treated patients (93%) without p53 mutations achieved a partial remission, only one of seven treated patients (14%) with p53 mutations achieved a partial remission (P = .00009). Adjusting for prognostic factors (age, sex, race, and Rai stage), patients with p53 gene mutations had a 13-fold greater risk of death than patients without p53 mutations (P = .013). In addition to examining the clinical relevance of p53 gene mutations in B-CLL, we investigated the possible role of p53 gene regulation in the expression of the multidrug resistance genes MDR1 and MDR3. We quantitated MDR1 and MDR3 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR). Expression of both the MDR1 and MDR3 genes was independent of p53 gene mutation or prior drug treatment, and did not predict for clinical response. Our findings indicate that p53 gene mutations in B-CLL are associated with a poor clinical outcome and may be a prognostic indicator for drug resistance.
### Table 1. Summary of Clinical Parameters of 53 B-CLL Patients

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<th>Patient No.</th>
<th>Age at Diagnosis (yr)</th>
<th>LDT (mo)</th>
<th>Rai Stage</th>
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### Patients without p53 mutations

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**Abbreviations:** NA, not assessable; IC<sub>50</sub>(CLB), concentration of CLB in μmol/L that inhibits viability of 50% of the lymphocytes in vitro; C, cytoxan (Bristol-Myers, Evansville, IN); CAE, Cytoxan + Adriamycin + etoposide-16; CLB, chlorambucil; CP, chlorambucil + prednisone; CVP, cytoxan + vincristine + prednisone; CHOP, Cytoxan + Adriamycin + vincristine + prednisone; FLU, fludarabine; dCF, 2'-deoxycoformycin; M, mitoxantrone; E, etoposide; C + P, Cytoxan + prednisone; P, prednisone; SPX, splenectomy; NT, not tested before initial chemotherapy.
clear cells were isolated by centrifugation on a Ficoll-Hypaque gradient. B lymphocytes were further enriched by rosetting T cells with CD19 (clone 89B, IgG1k; Coulter, FL) and phycoerythrin-labeled mAb to CD14 (clone Cr3-6, IgG1; Olympus Corp., NY), respectively. Cells were incubated for 30 minutes with the appropriate dilution of the mAbs in ice-cold phosphate-buffered saline (PBS) containing 1% bovine serum and 1% human AB serum (GIBCO, BRL, NY). Cells were washed, resuspended in PBS, and then applied to an Ortho Cytofluorograph equipped with an argon ion laser (Ortho Instruments, MA). Sorted cells were collected into ice-cold RPMI with 20% fetal calf serum (FCS). High-molecular weight DNA was isolated by digestion with proteinase K (Boehringer, Indianapolis, IN), extraction with phenol/chloroform, and ethanol precipitation.  

In vitro chemosensitivity assay. B lymphocytes obtained from patients before initial therapy were cultured on microtiter plates (2.5 × 10^6 cells/well) in 180 μL of RPMI 1640 plus 10% FCS in the presence of chlorambucil or without the drug for 72 hours followed by addition of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described. The IC₅₀ is defined as that concentration of the drug (μmol/L) that inhibits viability by 50%.  

Oligonucleotide primers. Primers used to amplify exons 4 through 9 of the p53 gene have been previously described. Amplimers used for amplification of MDR1 and β-actin–specific sequences have also been reported. MDR3-specific sequences were amplified using the following primers: sense strand, GCTTCTGCAAATGGTTTGA (residues 2602 to 2621), and antisense strand, TCGAAAACACCGGCATAGG (residues 2851 to 2870). These primers generate a 268-kb PCR product. All oligonucleotide primers were either synthesized with an Applied Biosystems Synthesizer (model 380A) or purchased from OPERON (Technologies Inc., CA).  

Detection of p53 mutations. Mutations of p53 in B-CLL patients were detected by single-strand conformation polymorphism (SSCP) analysis of PCR products. Primer pairs for exons 4 through 9 were used to amplify p53 coding sequence, using 100 ng of genomic DNA isolated from the lymphocytes of each patient. The PCR mixture, the amplification conditions, and direct sequencing of PCR products performed with the sequencing kit (United States Biochemical) were described previously. When screening for mutations in the SSCP assay and DNA sequencing, genomic DNA from samples containing known wild-type and mutant p53 alleles were amplified by PCR and run in parallel as controls.  

Detection of gene expression by reverse transcription and PCR. Total cellular RNA was prepared by guanidine isothiocyanate extraction. Poly A + mRNA was purified by affinity adsorption to oligo(dT)-cellulose using the Fast Track mRNA isolation kit (In Vitrogen, CA). Complementary DNA (cDNA) was prepared by reverse transcription (RT) of mRNA (0.5 μg) from purified B lymphocytes or cell lines using 50 ng of random hexadeoxynucleotide primer and 200 U of MOLLY reverse transcriptase (SuperScript RNase H-Reverse transcriptase; BRL) under conditions recommended by the supplier. cDNA aliquots equivalent to 0.05 μg (β-actin) and 0.25 μg (MDR1 and MDR3) RNA were used for enzymatic amplification by PCR using 2 U of AmpliTAQ polymerase (Perkin-Elmer/Cetus, Norwalk, CT). PCR was performed in 50 μL containing 1 μmol/L of specific primers. Twenty-five cycles were performed; each cycle included 1 minute of denaturation at 94°C, 1 minute of primer annealing (55°C, MDR1; 57°C, MDR3; 60°C, β-actin) and 2 minutes of extension at 72°C. PCR products (20 μL/reaction) were analyzed by electrophoresis on 1.2% agarose gel and visualized after ethidium bromide staining. Gels were processed for Southern blotting. The membranes containing the DNA fragments were hybridized with specific oligonucleotides end-labeled with [γ-32P]-ATP, washed, and exposed to x-ray film at –70°C for 2 hours: MDR1, ACTAGAAGGTCGCTGGAAAG; MDR3, GGACAGTTGTGCTTGGAC; β-actin, GGAGTCCTGTCGACATCCAC. The hybridization signals were quantified by densitometric scanning of the autoradiographs (LKB Ultrascan XL, Laser Densitometer). The human leukemia cell line CCRF-CEM and its vinblastine-resistant derivative CEM/VLB100 served as negative and positive controls, respectively, for MDR1 gene expression. The human liver cell line HepG2 served as a positive control for MDR3 gene expression and the CEM/VBL100 cell line was used as the negative control.  

Statistical analysis. Comparison between patients with p53 status was assessed using the Mann-Whitney test (continuous variables) and Fisher’s exact test (nominal variables). Survival distributions for the two groups of patients were compared using the Kaplan-Meier method. The difference between the two survival curves was assessed using Gehan’s generalized Wilcoxon test. To control for the effects of confounding variables, ie, prognostic factors that differed by p53 status, the data were subjected to the Cox proportional hazards model. Using this procedure, a time-weighted relative risk for death was estimated and was adjusted for pertinent confounders. P values in all cases were two-tailed. All statistical calculations were performed using SAS procedures.  

RESULTS  

Identification of p53 mutations. Mutations of p53 were detected in B lymphocytes from eight of 53 patients (15%) by SSCP analysis (Fig 1). At the time of p53 gene analysis, six of these eight patients had been untreated. Mutations were confirmed by DNA sequencing (Fig 2) and the data are listed in Table 2. Six of the mutations were missense mutations, resulting in an amino acid substitution, and two of the mutations were nonsense mutations, resulting in premature termination of protein translation. The mutations were located in the highly conserved domains of the p53 gene. Three mutations occurred in exon 5 (37%), two in exon 6 (25%), and one each in exons 4, 7, and 8.  

Patient characteristics and clinical course. The 45 patients without p53 mutations had a median age at diagnosis of 62 years (range, 36 to 80) and the eight patients with mutations had a median age of 67 (range, 49 to 81) (P = NS). There was no difference in gender between the two groups. As shown in Table 1, no patient with a p53 gene mutation was in Rai stage 0 or I, compared with 57% of patients without p53 mutations (P = .002). A LDT of less than 8 months was observed in five of eight patients (63%) with p53 mutations, compared with only 9% of the assessable patients without mutations (P = .003).  

Of the 45 patients without p53 mutations, 29 (64%) required therapy, and 27 of these patients (93%) had a partial remission. In contrast, 7 of 8 patients (87%) with p53 mutations required therapy, but only one patient (14%) achieved a partial remission (P = .0009). A shorter survival duration
was also observed in patients with p53 mutations (Fig 3). Six of eight patients (75%) with p53 mutations have died, compared with eight of 45 patients (18%) without p53 mutations (relative risk, 4.66; \( P = .15 \) by Gehan’s generalized Wilcoxon test). Adjusting for prognostic factors (age, sex, race, and Rai stage), patients with p53 gene mutations had a 13-fold greater risk of death than patients without p53 mutations (\( P = .013 \)).

**Chemosensitivity assays.** Before receiving chemotherapy, the B lymphocytes from 28 patients (six with p53 mutations) were tested for drug resistance with the MTT chemosensitivity assay (Table 1). Because IC50 values are significantly increased following chlorambucil treatment,45

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

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* Six of eight patients did not receive chemotherapy before DNA analysis for p53 gene mutations.
IC$_{50}$ data as a predictive indicator are presented only for those patients who were tested before therapy. The mean IC$_{50}$ of chlorambucil was 3.4-fold greater when p53-positive lymphocytes were tested compared with p53-negative lymphocytes ($P = .006$).

**MDR1 and MDR3 gene expression.** RNA samples obtained from B lymphocytes of 16 B-CLL patients were analyzed for coexpression of the MDR1, MDR3, and $\beta$-actin genes using the RT-PCR method. The results of Southern blot analysis of gene expression for MDR1, MDR3, and $\beta$-actin are shown in Fig 4. The oligonucleotides used to probe the Southern blots for either the MDR1 or the MDR3 gene were specific and hybridized only with their respective cDNA products. Seven of the patients had p53 mutations (lanes a through g). Of the nine patients without p53 mutations (lanes h through p), six had received drug treatment (lanes h through m), while three other patients were without drug treatment (lanes n through p). mRNA from human cell lines specific for MDR1 (CEM/VBL100) and MDR3 (HepG2) gene expression served as positive controls (lanes 1). mRNA from human cell lines with basal levels of MDR1 (CCRF-CEM) and MDR3 (CEM/VBL100) expression served as negative controls (lanes 2). Measurement of $\beta$-actin gene expression was used as an internal control for RNA recovery from each patient. Densitometric quantitation of MDR1 and MDR3 gene expression of B lymphocytes from normal healthy donors are summarized in Table 3.

The drug-resistant cell line CEM/VBL100 expressed a 12-fold greater level of the MDR1 gene relative to the parental drug-sensitive CCRF-CEM cell line. A twofold increase in MDR1 expression was observed in sorted CD19$^+$ and CD14$^+$ cells from normal healthy donors (Table 3). All 16 patients analyzed for MDR1 gene expression showed an intermediate to high expression (Fig 4 and Table 3). There was no association between MDR1 expression and the following: p53 gene mutations, prior drug treatment, clinical course of the disease, and Rai stages.

MDR3 gene expression was sevenfold greater in the human liver cell line HepG2 relative to the negative cell line CEM/VBL100. A twofold to threefold increase in MDR3 expression was observed in sorted CD19$^+$ cells and no expression was observed in CD14$^+$ cells from normal healthy donors (Table 3). Of 16 patients analyzed for MDR3 gene expression (Fig 4 and Table 3), 12 expressed the MDR3 gene. Eight patients showed intermediate to high levels, four showed low expression (absorbance value less than sorted CD19$^+$ cells), and four showed no expression of the MDR3 gene. Intermediate to high expression of the MDR3 gene was detected in 43% of patients with p53 mutations and 56% of patients without p53 mutations (Table 3). There was no association between prior drug treatment, Rai stage, clinical response to treatment, and MDR3 gene expression. In addition, there was no association between the levels of expression of MDR3 and MDR1 in the same patient.

**DISCUSSION**

Mutations in the p53 tumor suppressor gene were identified in eight of 53 B-CLL patients. It is less likely that chemotherapy selected out p53 mutant clones of B lymphocytes, since six of eight patients with p53 mutations were untreated at the time of DNA analysis. In the other two patients, DNA was not available before treatment. Patients with p53 gene mutations differed from those without mutations in several ways: they were more likely to be in Rai stage II or higher, to have a decreased LDT, to require chemotherapy, to show resistance to chlorambucil in vitro, to have a poor clinical response to therapy, and to have a shorter survival duration.

In view of the report that p53 mutations may be involved in regulation of the MDR1 gene,4 we examined if p53 mutations were associated with modulation of the genes involved in multidrug resistance in B-CLL patients. The B lymphocytes from all patients expressed high levels of the MDR1 gene. MDR1 gene overexpression was not dependent on the presence of p53 mutations, suggesting that MDR1 overexpression in B-CLL lymphocytes is not regulated by the p53 gene product. Similarly, it has been recently suggested that MDR1 in AML expression is not affected by the presence of p53 mutations.45

Overexpression of MDR1 has been associated with resistance to chemotherapy in many malignancies.47 Drugs used for ANLL therapy, such as the anthracyclines, are susceptible to the action of the P-170 efflux pump. In untreated ANLL, 67% of patients with high MDR1 levels fail to go into complete remission after one cycle of anthracycline-based treatment, compared with 33% of ANLL patients with low MDR1 expression.17 In a prospective study of 122 patients with untreated ANLL, 68% of P-170–positive patients did not achieve complete remission compared with 19% of the P-170–negative patients.18 In our study, the absence of any association between MDR1 expression and
Fig 4. Southern blot analysis of gene expression by RT-PCR of RNA from B-CLL patients. The coexpression of MDR1, MDR3, and β-actin genes was analyzed in 16 B-CLL patients (lanes a-g, patients no. 1-7; lanes h-m, patients no. 9-14; lanes n-p, patients no. 38-40) whose clinical parameters are detailed in Table 1. mRNA from human cell lines CEM/VLB100 and HepG2 served as positive controls (lane 1) for the expression of MDR1 and MDR3 genes, respectively. mRNA from human cell lines with basal levels of MDR1 (CCRF-CEM) and MDR3 (CEM/VLB100) served as negative controls (lane 2) for MDR1 and MDR3 gene expression, respectively. PCR-amplified products were separated on agarose gels, Southern blotted and hybridized to 32P-labeled oligonucleotide probes specific for each gene product. Films were exposed for 2 hours at −70°C. The signals on the autoradiographs were scanned by a laser densitometer to quantitate the relative levels of gene expression for each patient (see Table 3). The expression of β-actin served as an internal control for the recovery of RNA from each patient.

clinical response to therapy may be due to the fact that most of the drugs used to treat B-CLL are not affected by the MDR1 gene product, P-170.

Our results show intermediate to high levels of MDR1 gene expression in B lymphocytes from all 16 B-CLL patients tested. High expression of the MDR1 gene in B lymphocytes from 90% to 100% of B-CLL patients has been shown in two other studies. In contrast, we and others found low expression of MDR1 in sorted CD19+ B lymphocytes from normal healthy donors. Similar results of low MDR1 expression have been reported for enriched populations of normal B lymphocytes. All of the studies to date show that MDR1 expression is usually low in B lymphocytes obtained from normal donors compared with B lymphocytes obtained from B-CLL patients. Further studies on MDR1 expression in CD5+B lymphocytes from normal donors and B-CLL patients are needed to understand this difference in the pattern of MDR1 gene expression and its role in the disease.

MDR3 is the second human multidrug resistance gene to be identified by its homology with the MDR1 gene. A physiological role of MDR3 in the phenomenon of multidrug resistance is not yet established. In this study, we observed a low expression of MDR3 in normal sorted CD19+ cells and highly variable MDR3 gene expression in B-CLL patients. Previous studies have showed high levels of MDR3 expression in untreated patients with prolymphocytic leukemia, or in patients in advanced Rai stages III and IV. Our study showed no association between the level of MDR3 expression and Rai stage, previous drug treatment or the presence of p53 gene mutations. Additional studies of MDR3 expression in a large group of B-CLL patients will be required to determine whether there is an association with any of the clinical parameters.

This report demonstrates an association between p53 gene mutations and an aggressive form of B-CLL characterized by advanced Rai stage, rapid LDT, increased drug resistance as measured by a poor response to chemotherapy, and shortened survival duration. Mutations of the p53 gene have been associated with disease progression and decreased survival in many other human cancers, including colon adenocarcinoma, breast cancer, CML, multiple myeloma, transitional cell bladder cancer, gastric cancer, and lung cancer. No other studies to date have linked the presence of p53 mutations in B-CLL with decreased response to chemotherapy, in vitro drug resistance, and a shortened survival duration. Our results suggest that p53 mutations may become a marker for drug resistance in B-CLL. Since the
Patients with p53 mutations

Controls
CEM/VBL100
2.4
0.2
CEM
0.2
0.6
HeptG2
ND
1.5
CD19\(^+\) cells
Donor 1
0.4
0.5
Donor 2
0.5
0.6
Donor 3
0.1
0.0
CD14\(^+\) cells
Donor 1
0.1
0.0
Donor 2
0.1
0.0

Patients with p53 mutations

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<th>MDR3</th>
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Abbreviations: NT, not treated; ND, not determined.
* The values given are the difference between the minimum and maximum absorbance of each sample.

overall frequency of p53 mutations is relatively low in B-CLL, further studies in a larger population of patients will be needed to establish the reliability of a p53 mutation as a prognostic indicator.

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p53 MUTATION AND DRUG RESISTANCE IN B-CLL


p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression

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