

ORIGINAL ARTICLE

ZAP-70 Compared with Immunoglobulin Heavy-Chain Gene Mutation Status as a Predictor of Disease Progression in Chronic Lymphocytic Leukemia

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ABSTRACT

BACKGROUND

The course of chronic lymphocytic leukemia (CLL) is variable. In aggressive disease, the CLL cells usually express an unmutated immunoglobulin heavy-chain variable-region gene (*IgV_H*) and the 70-kD zeta-associated protein (ZAP-70), whereas in indolent disease, the CLL cells usually express mutated *IgV_H* but lack expression of ZAP-70.

METHODS

We evaluated the CLL B cells from 307 patients with CLL for ZAP-70 and mutations in the rearranged *IgV_H* gene. We then investigated the association between the results and the time from diagnosis to initial therapy.

RESULTS

We found that ZAP-70 was expressed above a defined threshold level in 117 of the 164 patients with an unmutated *IgV_H* gene (71 percent), but in only 24 of the 143 patients with a mutated *IgV_H* gene (17 percent, $P < 0.001$). Among the patients with ZAP-70-positive CLL cells, the median time from diagnosis to initial therapy in those who had an unmutated *IgV_H* gene (2.8 years) was not significantly different from the median time in those who had a mutated *IgV_H* gene (4.2 years, $P = 0.07$). However, the median time from diagnosis to initial treatment in each of these groups was significantly shorter than the time in patients with ZAP-70-negative CLL cells who had either mutated or unmutated *IgV_H* genes ($P < 0.001$). The median time from diagnosis to initial therapy among patients who did not have ZAP-70 was 11.0 years in those with a mutated *IgV_H* gene and 7.1 years in those with an unmutated *IgV_H* gene ($P < 0.001$).

CONCLUSIONS

Although the presence of an unmutated *IgV_H* gene is strongly associated with the expression of ZAP-70, ZAP-70 is a stronger predictor of the need for treatment in B-cell CLL.

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THE CLINICAL COURSE OF CHRONIC lymphocytic leukemia (CLL) is variable.¹⁻³ Some patients have aggressive disease and require therapy within a relatively short time after diagnosis, whereas others have indolent, asymptomatic disease, need no therapy for many years, and are not likely to benefit from palliative chemotherapy.⁴ Because of the difficulty of predicting the course of disease at the time of diagnosis and the uncertainty of the value of early treatment, therapy is currently recommended only for patients with disease that is progressive, symptomatic, or both.^{5,6} This practice continues despite the advent of new treatments that might be effective in patients with a low tumor burden.

CLL cells may have several characteristics that are associated with relatively aggressive disease. One such characteristic is the absence of somatic mutations in rearranged *IgV_H* genes. (Germ-line gene segments, termed *V_H*, *D*, and *J_H*, rearrange to form a gene that encodes the variable region of the heavy chain of the immunoglobulin molecule; somatic mutations in these segments typically occur in antigen-stimulated B cells.) Others are functional loss of *p53*, expression of CD38, the del(11q22.3) mutation, or complex cytogenetic abnormalities.^{7,8} Currently, unmutated *IgV_H* genes are the strongest predictor of aggressive disease.⁹⁻¹⁴ However, aggressive disease is not always associated with a rearranged *IgV_H* gene that is unmutated.^{15,16} Furthermore, sequencing of rearranged *IgV_H* genes is not easily performed in a clinical laboratory.

DNA-microarray studies have shown that the gene-expression patterns of CLL cells with unmutated *IgV_H* genes are similar to those of cells with mutated *IgV_H* genes, but that the patterns of both are distinct from those of other leukemias and lymphomas.^{17,18} Nevertheless, the two subtypes of CLL can be distinguished by the differential expression of a small number of genes, one of which encodes ZAP-70, an intracellular tyrosine kinase with a critical role in T-cell-receptor signaling.^{19,20} Recent studies have found that ZAP-70 is associated with enhanced signaling by the cell-surface immunoglobulin receptor of CLL B cells, irrespective of the mutational status of *IgV_H*,²¹ and that measurement of ZAP-70 can serve as a surrogate for the mutational status of *IgV_H*.^{22,23}

We sequenced the *IgV_H* genes and measured ZAP-70 levels in CLL B cells from 307 patients who are followed by the Chronic Lymphocytic Leukemia Research Consortium (CRC) to examine whether

the expression of ZAP-70 by CLL B cells is a stronger predictor of the need for early treatment than *IgV_H* mutation status.

METHODS

SAMPLE PROCESSING AND PATIENT SELECTION

Written informed consent was obtained from all the patients at the time of their enrollment in the CRC. Blood was drawn from 307 patients with CLL (Table 1) and 14 age-matched, healthy adults. The CRC institutions provided the date of diagnosis and the date of the initiation of therapy, if therapy had been given. The samples were analyzed to determine both the ZAP-70 level and the *IgV_H* mutational status. Peripheral-blood mononuclear cells were isolated by density-gradient centrifugation with the use of Ficoll-Paque Plus (Amersham Biosciences). The isolated cells were washed and then suspended in fetal-calf serum containing 10 percent dimethylsulfoxide for storage in liquid nitrogen for subsequent use.

CLINICAL DATABASE

Clinical and basic-science data related to each blood sample were collected with use of the CRC Information Management System, a 128-bit encrypted, password-secured Web application. The system allows CRC investigators to enter and review correlative clinical data pertaining to blood samples sent to the tissue core laboratory. Analyses of samples accessioned through the tissue core laboratory are available to laboratory staff and CRC investigators through role-specific access controls (i.e., predefined levels of access for each user's role within the CRC). Patient data and samples are all de-identified by means of masked serial identifiers. All activities regarding the use of patient data and samples followed or exceeded the requirement guidelines set forth in the Health Insurance Portability and Accountability Act.

ANALYSIS FOR ZAP-70

Immunoblot analyses for ZAP-70 were performed as previously described²¹ in CLL B cells isolated to greater than 99 percent purity with the use of magnetic beads coupled to monoclonal antibodies specific for CD19 (DynaL Biotech). For flow cytometry, peripheral-blood mononuclear cells were stained for 20 minutes at 4°C with CD19-specific and CD3-specific monoclonal antibodies conjugated with allophycocyanin and phycoerythrin, respectively

(Pharmingen). The cells were washed twice and fixed with 4 percent paraformaldehyde in phosphate-buffered saline and then permeabilized with saponin in Hanks' balanced salt solution for five minutes at 4°C. The cells were washed and then stained for 45 minutes at 4°C with a monoclonal antibody specific for ZAP-70 (clone 1E7.2) that had been conjugated to Alexa-488 dye (Becton Dickinson).

The samples were washed and analyzed by flow cytometry (FACSCalibur, BD Biosciences) and FlowJo software, version 2.7.4 (Tree Star). Lymphocytes were gated on the basis of their forward-angle light scatter and side-angle light scatter. Quadrants were set on gated cells such that 0.1 percent of the total lymphocytes were in the upper right quadrant. This gating was used for all the subsequent samples in the experiment. The expression of ZAP-70 was measured by calculating the percentage of CD19+CD3− cells that was above this gating threshold. In each experiment, we used control cells from the healthy donors, CLL cells from one patient with high levels of ZAP-70, and CLL cells from another patient with low levels of ZAP-70. CLL cells also were analyzed for CD19, CD20, and CD23 with the use of monoclonal antibodies conjugated to allophycocyanin, peridinin–chlorophyll–A–protein complex, and fluorescein isothiocyanate, respectively (Pharmingen), as previously described.² Fluorochrome-conjugated, isotype control monoclonal antibodies of irrelevant specificity were used in all experiments to monitor for nonspecific staining.

SEQUENCE ANALYSIS OF EXPRESSED *IgV_H*

We isolated RNA from CLL cells with RNeasy (Qiagen). First-strand complementary DNA (cDNA) was synthesized from total RNA with the use of oligo-dT primers and SuperScript II (Life Technologies). The remaining RNA was removed with RNase H, and the cDNA was purified with QIAquick purification columns (Qiagen). The purified cDNA was poly-dG–tailed with deoxyguanosine triphosphate and terminal deoxytransferase (Roche). The *IgV_H* subgroup expressed by the CLL B cells was determined with the use of a reverse-transcription polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay.²⁴ The cDNA was amplified by PCR with the use of a mixture of sense-strand oligonucleotide primers specific for the leader sequence of the expressed antisense oligonucleotide primer specific for the μ heavy-chain constant region. The PCR products were selected according to

Table 1. Characteristics of the Patients.

Characteristic	Value
Male sex — no. of patients (%)	201 (65)
Age at diagnosis — yr	
Median	52
Range	30–77
Therapy begun	
No	
No. of patients	155
Time since diagnosis — yr	<1–17
Yes	
No. of patients	152
Time between diagnosis and therapy — yr	0–26
ZAP-70 level	
0% to 10%	123 (40)
>10% to \leq 20%	43 (14)
>20% to \leq 30%	30 (10)
>30%	111 (36)
<i>IgV_H</i>	
Unmutated (\geq 98% homology)	164 (53)
Mutated (<98% homology)	143 (47)

size by electrophoresis in 2 percent low-melt agarose. The products were then excised and purified (MinElute PCR Purification Kit, Qiagen). An upstream C μ oligonucleotide was used for priming the cDNA for fluorescence dideoxy chain-termination synthesis.

The cDNA fragments were evaluated with an automated nucleic acid–sequence analyzer (377, Applied Biosystems). Nucleotide sequences were analyzed with the use of DNASTAR software and compared with sequences in V Base and GenBank databases. The percentage homology to the closest germ-line *IgV_H* sequence was calculated from the number of nucleotide differences between the 5' end of framework 1 and the 3' end of framework 3. Sequences with less than 98 percent homology to the corresponding germ-line *IgV_H* sequence were considered mutated.

STATISTICAL ANALYSIS

Associations between ZAP-70 and *IgV_H* mutational status were assessed with the use of Fisher's exact test for binary data, the Kruskal–Wallis test for ordered categorical data, and the Wilcoxon rank-sum test for continuous data. ZAP-70 expression was considered both as a continuous variable and as an ordered categorical variable based on the following groups: 0 to 10 percent, greater than 10 percent but

no more than 20 percent, greater than 20 percent but no more than 30 percent, and greater than 30 percent. The time from a diagnosis of CLL to initial treatment was estimated by the method of Kaplan and Meier and assessed by the log-rank test. Data from patients who had not yet received treatment for the disease were regarded as censored. The association between the *IgV_H* mutational status, the expression of ZAP-70, and the time from the diagnosis to the initiation of therapy was investigated by means of a Cox proportional-hazards model. All P values are two-sided, and there is no correction for multiple comparisons.

RESULTS

DETECTION OF ZAP-70 BY FLOW CYTOMETRY AND IMMUNOBLOT ANALYSES

We detected ZAP-70 in purified CLL B cells (defined as CD19+CD3⁻ cells) by flow cytometry and immunoblot analyses (Fig. 1). A mean (\pm SD) of 1.3 ± 0.9 percent of the CD19+CD3⁻ B cells from the 14 healthy adults registered above the ZAP-70 threshold gate (Fig. 1C). The same gating strategy was applied to the CD19+CD3⁻ B cells from the 307 patients with CLL. The percentage of such cells containing ZAP-70 above the threshold was considered the ZAP-70 expression level. Representative dot blots of CLL samples with negligible, low, intermediate, or high levels of ZAP-70 are shown in Figure 1D, 1E, 1F, and 1G. The levels of ZAP-70 observed on flow cytometry correlated with the levels detected in isolated CLL cells on immunoblot analysis (Fig. 1H).

Using flow cytometry we observed a continuum in the levels of ZAP-70 expressed by CLL cells in the 307 samples, ranging from 0.3 percent to 98 percent (Fig. 2A). In 123 samples (40 percent), no more than 10 percent of the CD19+CD3⁻ cells were above the ZAP-70 gating threshold; in 43 samples (14 percent), more than 10 percent but no more than 20 percent were above the threshold; in 30 samples (10 percent), more than 20 percent but no more than 30 percent exceeded the threshold; and in 111 samples (36 percent), more than 30 percent of the CD19+CD3⁻ cells exceeded the ZAP-70 gating threshold.

We repeated the experiment four times in 8 of the 307 samples (Fig. 2C). In each experiment, the level of ZAP-70 detected in any one sample was in the same range as that initially assigned to that sample — that is, those with no more than 10 percent,

greater than 10 percent but no more than 20 percent, greater than 20 percent but no more than 30 percent, or greater than 30 percent of the CLL cells above the ZAP-70 threshold. Furthermore, in experiments conducted with samples from 10 patients, the levels of ZAP-70 did not change in serial samples obtained from the same patient over time (Fig. 2D). Finally, in experiments conducted with samples from three patients, the levels of ZAP-70 in CLL cells isolated from the blood did not differ from those in CLL cells obtained from bone marrow.

ASSOCIATION BETWEEN ZAP-70 AND TIME TO INITIAL THERAPY

Patients were treated when symptomatic or progressive disease developed, according to National Cancer Institute Working Group criteria.⁵ Of the 307 patients we studied, 152 had initiated therapy before enrollment (Table 1).

We examined the relationship between ZAP-70 expression and the time from diagnosis to initial therapy (Fig. 3). The median times from diagnosis to initial treatment in the patients who had CLL cells with ZAP-70 levels of 0 to 10 percent (9.2 years) and in the group with ZAP-70 levels of greater than 10 percent but no more than 20 percent (9.0 years) were not significantly different ($P=0.23$). Similarly, the median times from diagnosis to initial therapy in the group with ZAP-70 levels greater than 20 percent but no more than 30 percent (3.2 years) and the group with ZAP-70 levels exceeding 30 percent (2.6 years) were not significantly different ($P=0.70$). Recursive partitioning methods confirmed that a ZAP-70 level of 20 percent was an optimal threshold for classifying patients as ZAP-70-positive, in agreement with prior studies.²³ This threshold was identified regardless of the patients' age and in both men and women. Using this threshold, we found that the median time from diagnosis to initial therapy in the group of patients who were ZAP-70-positive (2.9 years) was significantly shorter than that in the group of patients who were ZAP-70-negative (9.2 years, $P<0.001$).

ASSOCIATION BETWEEN ZAP-70 AND *IgV_H* MUTATIONAL STATUS

The sequences of the *IgV_H* gene in each patient were in frame and functional. The CLL cells of 164 patients (53 percent) had unmutated *IgV_H* genes (i.e., they had 98 percent or greater sequence homology with germ-line *IgV_H* genes); in 14 patients (5 percent) the *IgV_H* genes had 96 to 98 percent homology

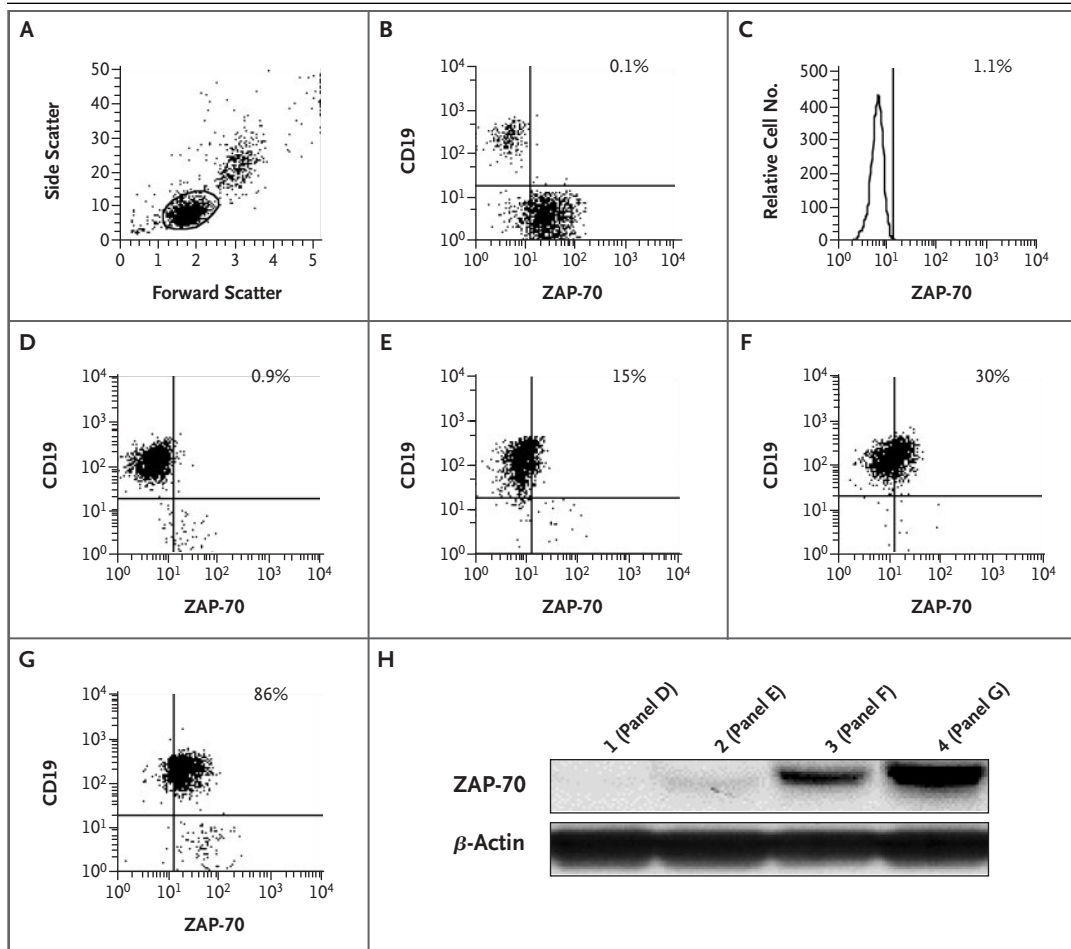
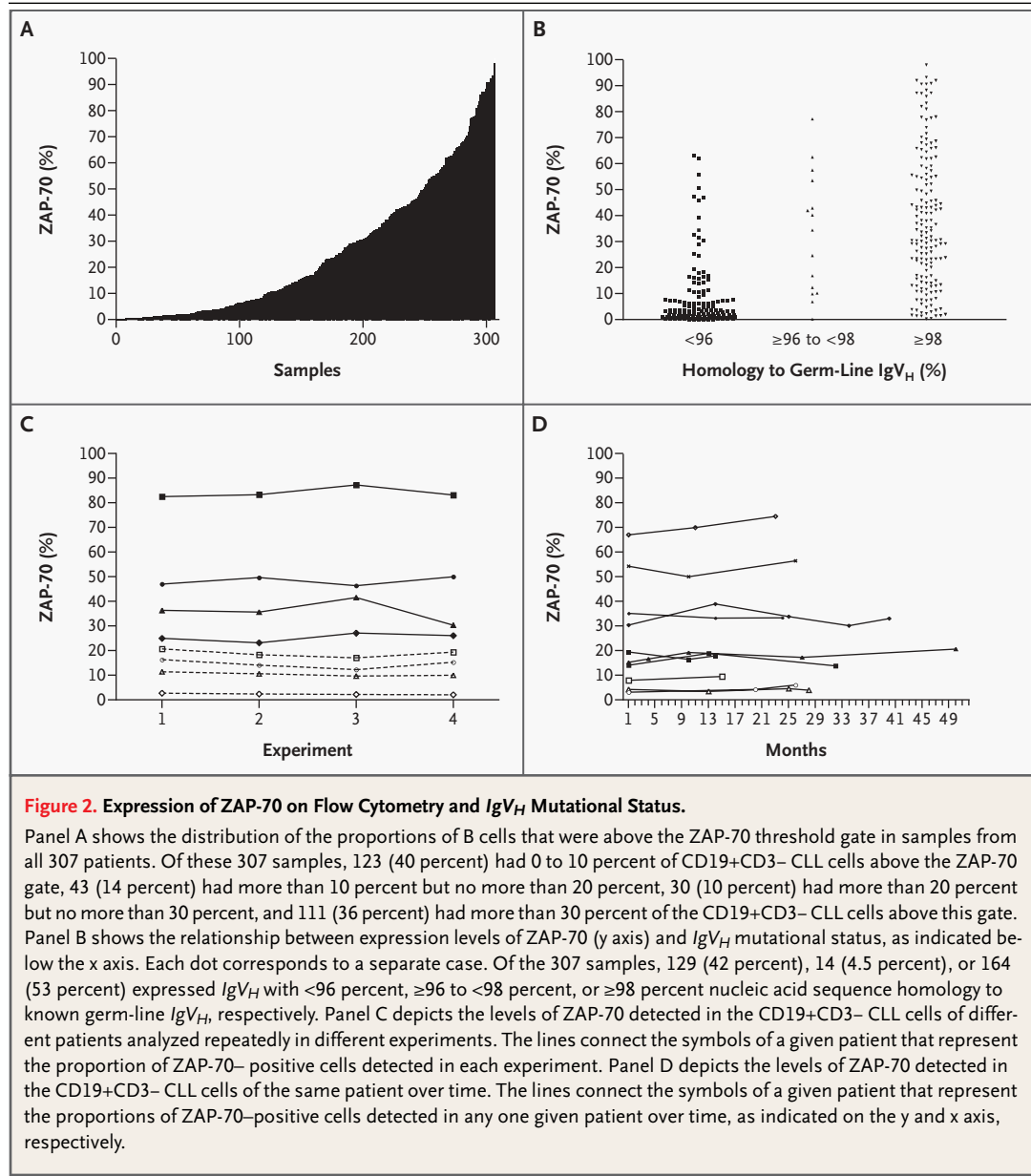


Figure 1. Detection of ZAP-70 in CLL B Cells by Flow Cytometry and Immunoblot Analysis.

Panel A shows a flow-cytometric plot of the forward-angle light scatter and side-angle light scatter of blood mononuclear cells from a healthy donor. A loop is drawn around the cells having the light-scatter characteristics of lymphocytes. Panel B shows a dot plot depicting the fluorescence of the lymphocytes gated in Panel A when stained with fluorochrome monoclonal antibodies specific for CD19 (on the y axis) and ZAP-70 (on the x axis). A threshold gate is set such that 0.1 percent of all the lymphocytes are in the upper-right quadrant. Panel C shows a histogram of staining for ZAP-70 in gated CD19+CD3⁻ B cells from a healthy adult. The percentage of CD19+CD3⁻ cells above the ZAP-70 threshold is indicated. Panels D, E, F, and G show representative histograms of staining for ZAP-70 in gated CD19+CD3⁻ CLL B cells from four patients; the percentages of the gated CD19+CD3⁻ lymphocytes above the ZAP-70 threshold are indicated. Panel H shows representative immunoblots of lysates of purified CD19⁺ CLL cells from four patients with CLL; the lysates were probed for expression of ZAP-70 or β -actin, as indicated. The samples in lanes 1, 2, 3, and 4 correspond to the analyses depicted in Panels D, E, F, and G, respectively. Lysates of isolated CLL cells that expressed negligible or low levels of ZAP-70 on flow cytometry (Panels D and E, respectively) did not have much detectable ZAP-70 protein by immunoblot analysis (lanes 1 and 2, respectively). Conversely, lysates of purified CLL cells that expressed intermediate or high levels of ZAP-70 on flow cytometry (Panels F and G, respectively) had readily detectable ZAP-70 protein on immunoblot analysis (lanes 3 and 4, respectively).

with germ-line genes; and 129 patients (42 percent) had mutated *IgV_H* genes in their CLL cells (*IgV_H* genes with less than 96 percent homology with germ-line *IgV_H* genes) (Table 2). Patients with CLL cells that expressed an unmutated *IgV_H* gene had a significantly shorter median time to initial therapy

(3.5 years) than patients with CLL cells that expressed a mutated *IgV_H* gene (9.2 years, $P < 0.001$). Regardless of the *IgV_H* subgroup examined, there was a significant association between the presence of unmutated *IgV_H* genes and ZAP-70 positivity (i.e., ZAP-70 levels above 20 percent) ($P < 0.001$). Of the



patients in whom the rearranged *IgV_H* gene had 98 percent or greater homology with a known germ-line *IgV_H* gene, 71 percent were ZAP-70-positive (Fig. 2B and Table 2); 83 percent of the patients with a mutated *IgV_H* gene were ZAP-70-negative. Nevertheless, 9 of the 141 ZAP-70-positive patients (6 percent) had an *IgV_H* gene with 96 to 98 percent homology with a known germ-line *IgV_H* gene, and 15 of them (11 percent) had an *IgV_H* gene with less than 96 percent homology. Conversely, 47 of the 166 ZAP-70-negative patients (28 percent) had an unmutated *IgV_H* gene.

ZAP-70 EXPRESSION, *IgV_H* MUTATIONAL STATUS, AND TIME FROM DIAGNOSIS TO INITIAL THERAPY

We compared all the patients according to both ZAP-70 expression and *IgV_H* mutational status and identified four groups. The median time from diagnosis to initial treatment among patients with ZAP-70-positive CLL cells that expressed an unmutated *IgV_H* gene was 2.8 years, which was similar to the 4.2 years among patients with ZAP-70-positive CLL cells that expressed a mutated *IgV_H* gene. The difference between these two groups was not significant ($P=0.07$). In the group of patients with ZAP-

70-negative CLL cells, the median times to initial therapy were 11.0 years among those with CLL cells that expressed a mutated *IgV_H* gene and 7.1 years among those with CLL cells that expressed an unmutated gene (Fig. 4); the difference was statistically significant ($P < 0.001$).

Cox proportional-hazards analysis of ZAP-70 expression and *IgV_H* mutation status as predictors of the time from diagnosis to initial therapy found that the hazard ratio associated with ZAP-70 positivity was 4.9 (95 percent confidence interval, 3.2 to 7.6), suggesting that the instantaneous risk of requiring therapy is 4.9 times as high among ZAP-70-positive patients as it is among ZAP-70-negative patients. Among patients with CLL cells that expressed an unmutated *IgV_H* gene, as compared with a mutated *IgV_H* gene, the hazard ratio was 2.5 (95 percent confidence interval, 1.6 to 3.9). This model suggests that patients with ZAP-70-positive CLL cells that express an unmutated *IgV_H* gene have a hazard of needing therapy that is 12.3 times that of patients with ZAP-70-negative CLL cells that express a mutated *IgV_H* gene.

DISCUSSION

In this study of CLL, we found a strong association between the expression of ZAP-70 in CLL cells (i.e., a ZAP-70 level above a defined threshold of 20 percent) and the unmutated *IgV_H* genes in agreement with the results of other studies.^{17,21-23,26} However, 23 percent of the 307 patients we studied had CLL cells that expressed mutated *IgV_H* and ZAP-70 or expressed unmutated *IgV_H* but lacked expression of ZAP-70. These discrepancies are apparently not related to the assay for ZAP-70, because we found good correlation between the results of flow cytometry and immunoblot analysis. Moreover, there appears to be little variation in the levels of ZAP-70 in the CLL cells of any one sample or in samples of any one patient over time. In previous studies of small numbers of patients, samples that were discordant with respect to ZAP-70 expression and *IgV_H* mutational status were occasionally found.²¹⁻²³ However, discordance between ZAP-70 and *IgV_H* mutational status becomes more apparent when larger numbers of patients are examined, as in this study.

The time from diagnosis to initial treatment is a useful clinical end point, since therapy for CLL is not currently initiated until progressive or symptomatic disease develops. In our series, patients who had more than 20 percent but no more than 30 percent

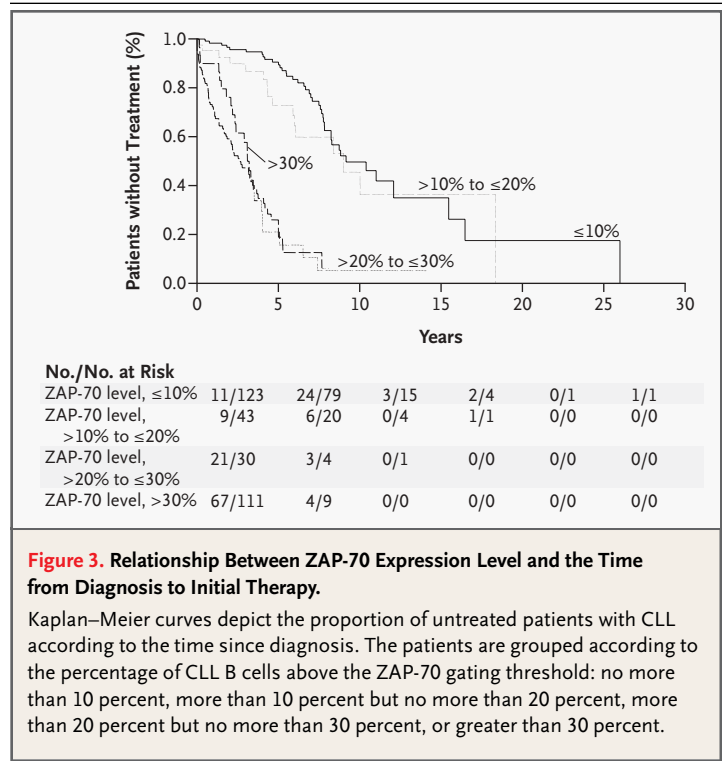


Figure 3. Relationship Between ZAP-70 Expression Level and the Time from Diagnosis to Initial Therapy.

Kaplan-Meier curves depict the proportion of untreated patients with CLL according to the time since diagnosis. The patients are grouped according to the percentage of CLL B cells above the ZAP-70 gating threshold: no more than 10 percent, more than 10 percent but no more than 20 percent, more than 20 percent but no more than 30 percent, or greater than 30 percent.

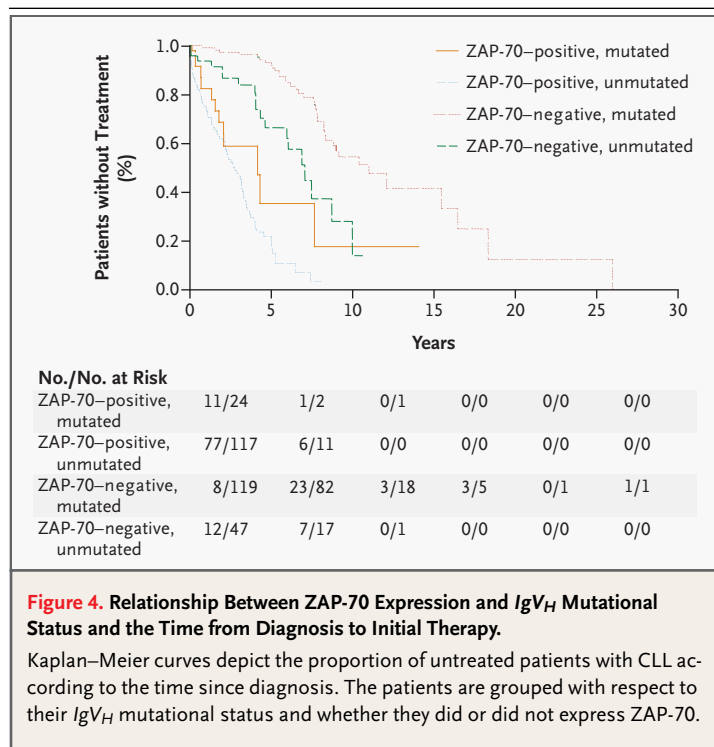
Table 2. *IgV_H* Expression and ZAP-70 Status.*

Variable	Percent Homology to Germ-Line <i>IgV_H</i>		
	≥ 98	≥ 96 to <98	<96
	no. of patients (%)		
<i>IgV_H</i> subgroup†			
<i>IgV_H1</i>	77 (47)	2 (14)	14 (11)
<i>IgV_H2</i>	2 (1)	0	15 (12)
<i>IgV_H3</i>	49 (30)	10 (71)	69 (53)
<i>IgV_H4</i>	35 (21)	2 (14)	30 (23)
<i>IgV_H5</i>	1 (1)	0	1 (1)
ZAP-70			
Positive	117 (71)	9 (64)	15 (12)
Negative	47 (29)	5 (36)	114 (88)
Total	164 (100)	14 (100)	129 (100)

* Because of rounding, not all percentages total 100.

† *IgV_H* genes are defined according to Kabat et al.²⁵

of CLL cells with ZAP-70 levels above the threshold and patients with more than 30 percent of CLL cells above the threshold had similar median times between diagnosis and treatment. Likewise, patients with no more than 10 percent of CLL cells above the ZAP-70 threshold and those with greater than



10 percent but no more than 20 percent of CLL cells above the threshold had similar median times from diagnosis to initial therapy. The median times from diagnosis to initial therapy in the latter two groups were significantly different from the times in the groups with more than 20 percent of CLL cells above the ZAP-70 threshold. These data support the use of 20 percent as the cutoff for defining ZAP-70 positivity. Using this cutoff, we found that the hazard ratio for the need for treatment in ZAP-70-positive patients, as compared with ZAP-70-negative patients, was 4.9. This ratio is greater than the hazard ratio of 2.5 associated with the presence of an unmutated *IgV_H* gene, which itself is also statistically significant.

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The reasons for the differences in clinical characteristics between patients with CLL cells containing a mutated *IgV_H* gene and those with an unmutated *IgV_H* gene are unknown. Examination of microarray data has shown that these two subtypes of CLL share a common gene-expression pattern, suggesting that they constitute a single entity.^{17,18} However, the microarray data have also revealed some important differences between the two types of CLL in the expression of a small number of genes. One or more of these genes might account for clinical differences more than does the mutational status of the *IgV_H* gene. ZAP-70 is an attractive candidate in this regard, owing to its participation in receptor signaling.^{19,20} Recent studies have found that ZAP-70 can take part in, and is associated with, increased immunoglobulin-receptor signaling in CLL cells.²¹ Such increased intracellular signaling could influence the survival or proliferation of CLL cells, leading to a tendency toward disease progression.

Our study shows that increased expression of ZAP-70 by CLL cells is a stronger predictor of the need for treatment than the presence of an unmutated *IgV_H* gene. Since flow cytometry can be used reliably to assess blood samples for ZAP-70, it should be more amenable for application in clinical laboratories than nucleic acid-sequence analyses of the rearranged *IgV_H* gene. Moreover, because the expression of ZAP-70 appears to be constant over time, it might be used at the time of diagnosis to identify patients who are at increased risk for early disease progression.

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