

ORIGINAL ARTICLE

ZAP-70 Expression as a Surrogate for Immunoglobulin-Variable-Region Mutations in Chronic Lymphocytic Leukemia

Marta Crespo, B.S., Francesc Bosch, M.D., Neus Villamor, M.D., Beatriz Bellosillo, Ph.D., Dolors Colomer, Ph.D., María Rozman, M.D., Silvia Marcé, B.S., Armando López-Guillermo, M.D., Elies Campo, M.D., and Emili Montserrat, M.D.

ABSTRACT

From the Department of Hematology (M.C., F.B., A.L.-G., E.M.) and the Hematopathology Unit (N.V., B.B., D.C., M.R., S.M., E.C.), Institut d'Investigacions Biomèdiques August Pi i Sunyer, Hospital Clínic, Barcelona, Spain. Address reprint requests to Dr. Bosch at the Department of Hematology, Hospital Clínic, Villarroel 170, Barcelona 08036, Spain, or at fbosch@clinic.ub.es.

Ms. Crespo and Drs. Bosch and Villamor contributed equally to the article.

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BACKGROUND

The mutational status of immunoglobulin heavy-chain variable-region (IgV_H) genes in the leukemic cells of chronic lymphocytic leukemia (CLL) is an important prognostic factor in the disease. We investigated whether the expression of ZAP-70 by CLL cells correlated with the IgV_H mutational status, disease progression, and survival.

METHODS

The expression of ZAP-70 was analyzed in T-cell and B-cell lines and in peripheral-blood samples from 56 patients with CLL with the use of flow cytometry, Western blotting, and immunohistochemistry. The results were correlated with the IgV_H mutational status and clinical outcome.

RESULTS

ZAP-70 was detected by flow-cytometric analysis in cells of T-cell lineage and in leukemic cells from 32 of 56 patients with CLL. In all patients in whom at least 20 percent of the leukemic cells were positive for ZAP-70, IgV_H was unmutated, whereas IgV_H mutations were found in 21 of 24 patients in whom less than 20 percent of the leukemic cells were positive for ZAP-70 ($P < 0.001$). Concordant results were obtained when ZAP-70 expression was assessed by immunohistochemistry or Western blotting. The level of ZAP-70 expression did not change over time (median, 37 months) in sequential samples from 30 patients with CLL. Patients with Binet stage A CLL who had at least 20 percent ZAP-70-positive leukemic cells had more rapid progression and poorer survival than those with less than 20 percent ZAP-70-positive cells.

CONCLUSIONS

Among patients with CLL, expression of ZAP-70, as detected by flow-cytometric analysis, correlated with IgV_H mutational status, disease progression, and survival.

THE STAGING SYSTEMS DEVELOPED BY Rai et al.¹ and Binet et al.² are standard methods of assessing prognosis in chronic lymphocytic leukemia (CLL). However, since these systems cannot identify stable or progressive forms of the disease, there has been a continual effort to identify other prognostic factors in CLL.³⁻⁶

About 50 to 70 percent of patients with CLL have evidence of somatic hypermutation in the immunoglobulin heavy-chain variable-region (*IgV_H*) genes of the leukemic cells.⁷⁻¹¹ These patients probably constitute a subgroup in whom the leukemic cells have passed through the germinal center, the site of *IgV_H* hypermutation.¹² It is important to note that patients with unmutated *IgV_H* genes usually have an advanced stage of CLL and unfavorable cytogenetic features, require therapy, and have a short survival. In contrast, patients with leukemic cells that have mutant *IgV_H* genes usually present in an early clinical stage, frequently have 13q14 chromosomal deletions, do not have alterations of p53, do not require therapy, and have a long survival.^{11,13,14} For these reasons, knowledge of the mutational status of *IgV_H* is of considerable value in assessing the prognosis in CLL. Most general laboratories, however, are unable to determine *IgV_H* sequences. Moreover, even when the technique is available, it is too costly and time consuming to include in the standard workup of CLL. These considerations have made finding a surrogate for *IgV_H* mutational status in CLL an important priority.

Investigations using DNA microarrays^{15,16} have shown that CLL cells exhibit a characteristic gene-expression profile in which the expression of a small subgroup of genes, including those encoding ZAP-70, IM1286077, and C-type lectin, correlates with the mutational status of *IgV_H* genes.^{16,17} ZAP-70, a member of the Syk-ZAP-70 protein tyrosine kinase family, is normally expressed in T cells and natural killer cells and has a critical role in the initiation of T-cell signaling.¹⁸⁻²² This finding led us to hypothesize that the expression of ZAP-70 could not only predict *IgV_H* mutational status but also serve as a prognostic factor in CLL. We therefore analyzed ZAP-70 protein in CLL cells from a series of patients using Western blotting, immunohistochemistry, and flow cytometry and correlated the results with the mutational status of the *IgV_H* genes and the clinical outcome.

METHODS

PATIENTS AND SAMPLE COLLECTION

Fifty-six patients who had received a diagnosis of CLL at our institution were selected on the basis of the availability of frozen samples for biologic studies. Lymph-node–biopsy specimens were available from eight patients. Progression was defined as a change to a more advanced clinical stage or the need for treatment. The time to progression and survival were calculated from the time of diagnosis. The median age was 60 years (range, 37 to 81), and the median duration of follow-up (from diagnosis) was 63 months.

Mononuclear cells from peripheral-blood samples were isolated on a Ficoll–Hypaque gradient (Seromed).²³ In three samples, CD19+ cells were isolated with use of anti-CD19 fluorescein isothiocyanate, followed by separation with anti-fluorescein isothiocyanate Microbeads (Miltenyi Biotec). Lymph-node–biopsy specimens from six additional patients who had received a diagnosis of mantle-cell lymphoma according to the criteria of the World Health Organization²⁴ were included in the analysis.

PREPARATION OF RNA, COMPLEMENTARY DNA, AND GENOMIC DNA

Total RNA was isolated with use of Ultraspec RNA (Biotech Laboratories) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 µg of RNA with the use of Moloney–murine leukemia virus reverse transcriptase (Invitrogen, Life Technologies). High-molecular-weight DNA was extracted from mononuclear cells isolated on a Ficoll gradient with use of a salting-out procedure.²⁵

AMPLIFICATION OF *IgV_H* GENES

We amplified cDNA using a set of six heavy-chain variable-region (*V_H*) family-specific primers (*V_H*1 through *V_H*6) that anneal to sequences in the leader region²⁶ along with primers complementary to the constant region (*IgM* and *IgG*).⁷ When amplification with these primers failed, an alternative set of primers specific to framework I region and the heavy-chain joining (*J_H*) region was used.²⁷ For the amplification of genomic DNA, approximately 100 ng of DNA was used in a total volume of 50 µl.¹¹

IgV_H MUTATIONAL STATUS

Polymerase-chain-reaction (PCR) products were purified either directly with use of the Concert rap-

id PCR purification system (GIBCO BRL), or by gel excision with use of the QIAEX II agarose-gel extraction kit (Qiagen). Products were directly sequenced from both strands with use of the Big Dye Terminator Cycle Sequencing Ready Reaction (versions 2.0 and 3.0, Applied Biosystems) according to the manufacturer's instructions. Sequencing analysis and alignments were performed with use of DNAPLOT software and the VBASE data library.²⁸ Samples in which fewer than 2 percent of base pairs differed from those of the consensus sequence were considered unmutated.⁸

WESTERN BLOTTING

Western blotting of cell lysates was carried out as previously described.²³ Cell lysates from the following cell lines were also included: Jurkat (a T-cell line derived from lymphoblastic lymphoma), JVM-2 (B-cell prolymphocytic leukemia), Granta 519 and REC (mantle-cell lymphoma), DHL-16 and DOHH-2 (follicular lymphoma), Ly1.2 and Ly3 (diffuse large-B-cell lymphoma), and Namalwa, Raji, Daudi, and Ramos (Burkitt's lymphoma). Blots were incubated with anti-ZAP-70 antibody (Upstate Biotechnology) and anti- α -tubulin (Calbiochem). Lysates from Jurkat cells, a T-cell line with a high level of expression of ZAP-70 protein,¹⁸ were used as a positive control. To analyze the influence of normal T cells present in the CLL samples, we mixed different concentrations of mononuclear cells from healthy blood donors with REC cells, a B-cell line that does not express ZAP-70.

IMMUNOHISTOCHEMISTRY

We also analyzed the expression of ZAP-70 in paraffin-embedded sections of lymph-node-biopsy specimens from eight patients with CLL and six patients with mantle-cell lymphoma using an anti-ZAP-70 antibody (ZAP-70-LR, Santa Cruz Biotechnology). We evaluated the level of ZAP-70 expression in tumor cells, T cells, and residual germinal-center areas.

FLOW CYTOMETRY

Mononuclear cells, as well as whole blood, from 56 patients with CLL and 10 healthy blood donors used as normal controls were fixed and permeabilized with use of the Fix & Perm kit (Caltag Laboratories) according to the manufacturer's instructions. Then 1.5 μ g of anti-ZAP-70 antibody per 500,000 cells was incubated for 20 minutes at room temperature, washed twice in phosphate-buffered saline (Biomérieux), incubated for 20 minutes with goat

antimouse immunoglobulin fluorescein isothiocyanate (Dako), washed, and then incubated with normal mouse serum for five minutes. Finally, CD3-phycoerythrin, CD56-phycoerythrin, CD19-peridinin chlorophyll protein cychrome 5.5, and CD5-allophycocyanine (BD Biosciences) were added, and the samples were incubated for 15 minutes. Samples were analyzed with a flow cytometer (FACS Calibur, BD Biosciences) with a gate on the fluorescence 2 detector to ensure that at least 1000 T cells and natural killer cells were analyzed in each sample. In samples from healthy control subjects, at least 5000 B cells were also analyzed. Analysis of stained samples was carried out with use of CellQuest software (BD Biosciences).

Lymphocytes were gated to avoid the inclusion of debris, monocytes, and doublets. The resultant cells were then gated to select CD3+CD56+ cells (T and natural killer cells), used as an internal control for ZAP-70 expression, and CD19+CD5+ (CLL cells). Biparametric dot graphs of cells that were stained for ZAP-70 and CD3 plus CD56 were independently plotted for T cells and natural killer cells and CLL cells. A marker that included T cells and natural killer cells (in the upper right quadrant of each graph) was used to calculate the percentage of CLL cells that were positive for ZAP-70. The percentage of CD19+ cells that also expressed CD38 was quantified as previously described.⁸

STATISTICAL ANALYSIS

Correlations between ZAP-70, CD38, and mutational status were analyzed with use of Wilcoxon's and Fisher's exact tests and a multiple regression analysis. To identify the level of ZAP-70 expression that could best be used to discriminate mutated from unmutated cells, we used a receiver-operating-characteristic plot.²⁹ Correlations between the clinical characteristics and the expression of ZAP-70 were also analyzed with use of Wilcoxon's or Fisher's exact test. Survival and time to progression were estimated according to the method of Kaplan and Meier and compared between groups by means of the log-rank test. All P values were two-sided, and the type I error was set at 5 percent. Statistical analyses were performed with use of SPSS software.³⁰

RESULTS

SOMATIC MUTATIONS IN IgV_H GENES

All sequences of IgV_H genes in the leukemic cells from the 56 patients were in-frame rearrangements, and only two premature stop codons were observed. We

found somatic mutations (up to 98 percent homology with germ-line sequences) in 21 of 56 patients (38 percent), including 7 that had 95.6 to 97.3 percent homology with germ-line sequences (Table 1).

WESTERN BLOT ANALYSIS OF ZAP-70 EXPRESSION

ZAP-70 expression was analyzed in 12 human cell lines corresponding to different stages of B-cell differentiation. Peripheral-blood lymphocytes from healthy blood donors (data not shown) and the Jurkat T-cell line were used as positive controls. The protein was not detected in any of the B-cell lines, except the Ramos cell line, in which it was expressed weakly (Fig. 1A).

Of 32 samples from the patients with CLL, 16 had a high level of expression of ZAP-70 on Western blotting and the remaining 16 had a low level of expression (Fig. 1B). The presence of more than 2 percent T cells in the blood samples influenced the result, especially when the proportion was 10 percent or higher (Fig. 1C). However, samples from all but two patients with CLL contained less than 15 percent T cells on Western blotting (mean \pm SD, 4.5 ± 5.5 percent); Patient 45 had more than 20 percent T cells in the sample.

IMMUNOHISTOCHEMICAL ANALYSIS OF ZAP-70 EXPRESSION

We analyzed lymph-node–biopsy specimens from eight patients with CLL, none of whom had *IgV_H* mutations, and six patients with mantle-cell lymphoma, which is characterized by proliferation of CD5+ B cells but not usually by *IgV_H* mutations. All lymph-node–biopsy specimens from the patients with CLL were positive for ZAP-70, with a diffuse pattern reflecting the infiltration by leukemic cells. T cells in the biopsy specimens showed stronger immunoreactivity than the CLL cells. Residual germinal centers were negative for ZAP-70. All mantle-cell lymphomas were negative for ZAP-70, whereas T cells in the tissue had a high level of expression of ZAP-70.

FLOW-CYTOMETRIC ANALYSIS OF ZAP-70 EXPRESSION

In samples of normal blood, flow-cytometric analysis disclosed a population with high and homogeneous expression of ZAP-70 (Fig. 2A). This population corresponded to T cells and natural killer cells. In these samples, the proportion of normal B cells (from healthy blood donors) expressing as much ZAP-70 as T cells and natural killer cells was 0 to 6.5 percent (mean, 4 ± 2.5). No significant differences

in ZAP-70 expression were observed between CD5– and CD5+ B-cell subpopulations (Fig. 2B).

The method used to quantify the percentage of CLL cells expressing as much ZAP-70 as T cells is shown in Figure 2C. Leukemic cells from 56 patients with CLL were analyzed for ZAP-70 expression by flow cytometry. In these samples, T cells and natural killer cells also displayed high and homogeneous levels of expression of ZAP-70. In contrast to normal CD5+ B cells, CD5+ CLL cells from 32 of 56 patients expressed high levels of ZAP-70 (Fig. 2D).

In all but one patient (Patient 45), there was complete concordance of ZAP-70 expression as assessed by flow cytometry, Western blotting, and immunohistochemistry (Table 1). These discrepant results in Patient 45 (Fig. 1B and Table 1) could be explained by the high number of T cells (20 percent) in the patient's sample. ZAP-70 expression was also analyzed in 30 of 56 patients from whom two sequential samples were available. The median time between the collections of the samples was 37 months (range, 11 to 64). None of these 30 patients had significant changes in ZAP-70 expression over time.

CORRELATION BETWEEN ZAP-70 PROTEIN AND *IgV_H* MUTATIONAL STATUS

There was a strong correlation between the presence of *IgV_H* mutations and the percentage of leukemic cells that expressed ZAP-70, as assessed by flow cytometry. In the receiver-operating-characteristic analysis, a value of 17.5 percent ZAP-70–positive CLL cells was the best cutoff for assigning *IgV_H* mutational status. We used a cutoff value of 20 percent for simplicity. Patients with unmutated *IgV_H* genes had higher percentages of ZAP-70–positive CLL cells than did patients with *IgV_H* mutations (48 ± 21 percent vs. 6 ± 4 percent, $P < 0.001$). Samples from all 21 patients with *IgV_H* mutations contained less than 20 percent ZAP-70–positive CLL cells, and all but 3 patients without *IgV_H* mutations (91 percent) had 20 percent or more ZAP-70–positive CLL cells (Fig. 3A and 3B). Conversely, all the patients with increased proportions of ZAP-70–positive cells did not have somatic mutations, and 88 percent of patients with low numbers of ZAP-70–positive cells had *IgV_H* mutations. Overall, the probability of the absence of somatic mutations in the presence of more than 20 percent ZAP-70–positive CLL cells (positive predictive value) was 100 percent (95 percent confidence interval, 89 to 100), whereas the probability of *IgV_H* mutations in the presence of a low percentage of ZAP-70–positive cells (negative pre-

Table 1. Main Biologic and Clinical Characteristics of the Patients.*

Patient No.	IgV _H			ZAP-70 Expression				CD38+ Cells	Stage at Diagnosis		Survival
	Somatic Mutations	Homology %	Family	No. of Nucleotide Changes	Western Blotting	Flow-Cytometric Analysis	IHC of Lymph-Node Biopsy Specimen		Binet	Rai	
1	No	100	4-39	0	—	69 (high)	—	99	A 0	29	
2	No	100	3-48	0	—	54 (high)	—	—	A I	10	
3	No	100	1-46	0	Strong	21 (high)	—	—	A 0	3†	
4	No	100	1-69	0	—	47 (high)	—	4	A I	61	
5	No	100	3-23	0	—	60 (high)	Strong	95	B II	74†	
6	No	100	1-69	0	—	59 (high)	Strong	—	B II	75†	
7	No	100	3-33	0	—	61 (high)	—	99	A I	60†	
8	No	100	1-03	0	—	45 (high)	Strong	—	B I	48†	
9	No	100	1-69	0	—	29 (high)	—	—	A 0	48	
10	No	100	1-03	0	—	64 (high)	—	46	A 0	58†	
11	No	100	1-69	0	—	64 (high)	—	94	A 0	58	
12	No	100	3-11	0	—	54 (high)	Strong	—	A 0	104	
13	No	100	1-18	0	—	70 (high)	—	1	A 0	77	
14	No	100	1-03	0	Strong	24 (high)	—	4	A 0	39†	
15	No	100	1-e	0	Strong	37 (high)	—	64	C IV	92	
16	No	100	3-11	0	Strong	46 (high)	—	30	A 0	26†	
17	No	100	1-69	0	Strong	46 (high)	—	11	A 0	84†	
18	No	100	1-e	0	Strong	30 (high)	—	95	A I	85†	
19	No	100	2-70	0	Strong	74 (high)	—	—	A 0	76†	
20	No	100	1-69	0	Strong	73 (high)	—	71	A 0	67†	
21	No	100	3-30	0	Strong	60 (high)	—	70	A I	80†	
22	No	100	3-74	0	Strong	30 (high)	—	80	B II	18	
23	No	99.7	1-69	1	—	35 (high)	Strong	83	A 0	67†	
24	No	99.6	4-34	1	Strong	77 (high)	—	50	A I	49†	
25	No	99.6	1-69	1	Strong	29 (high)	—	39	A I	92†	
26	No	99.3	1-69	2	Strong	73 (high)	Strong	53	A I	67	
27	No	99.3	3-49	2	—	73 (high)	Strong	99	A 0	78†	
28	No	99.3	1-69	2	Strong	51 (high)	—	95	A I	109†	
29	No	99	3-09	3	Strong	70 (high)	—	53	A 0	307	
30	No	98.6	3-48	4	—	71 (high)	—	99	A 0	19	

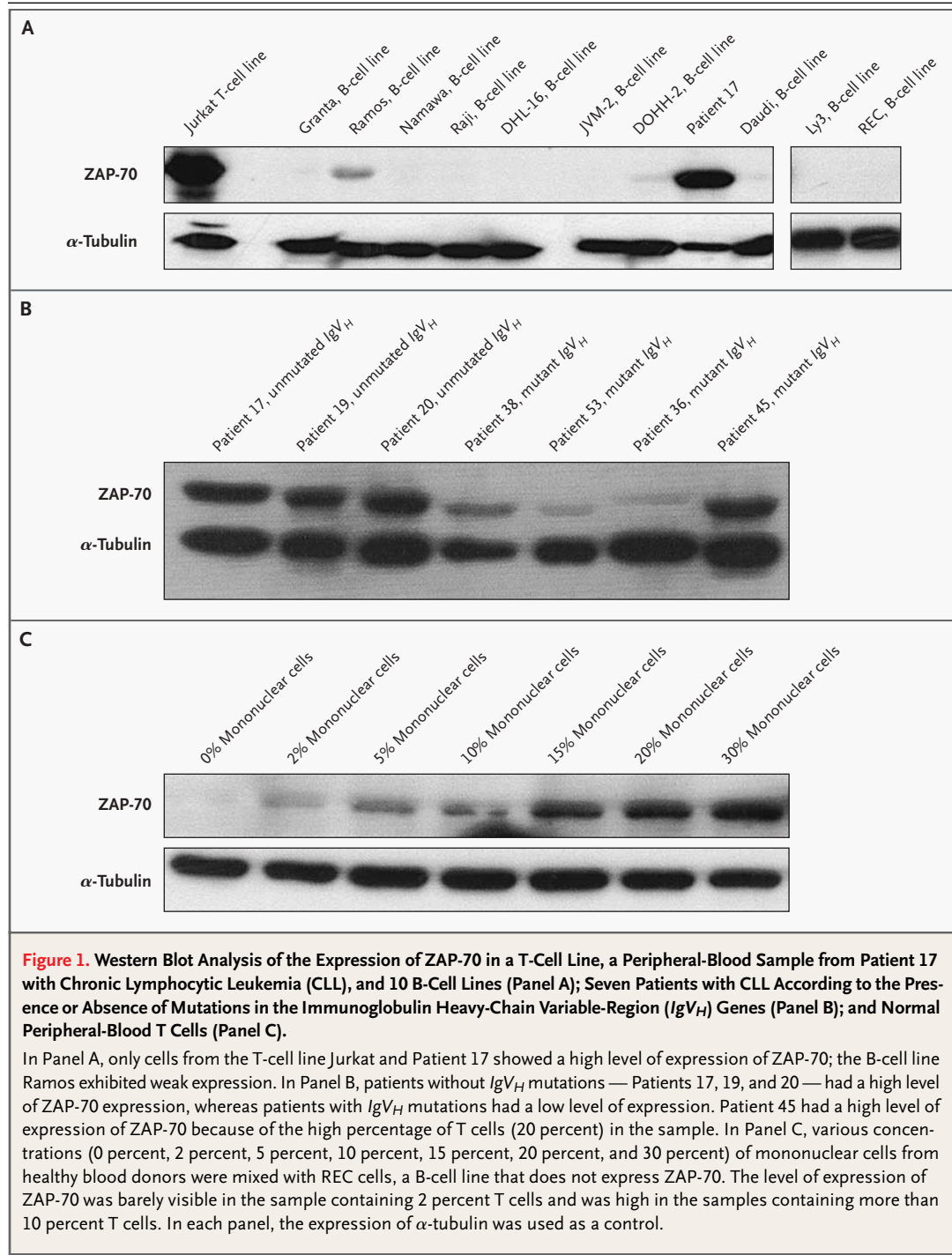
Table 1. (Continued.)

Patient No.	IgV _H			ZAP-70 Expression				CD38+ Cells	Stage at Diagnosis		Survival
	Somatic Mutations	Homology	Family	No. of Nucleotide Changes	Western Blotting	Flow-Cytometric Analysis	IHC of Lymph-Node Biopsy Specimen		Binet	Rai	
		%				% (category)				mo	
31	No	98.3	3-15	5	—	30 (high)	—	78	A	I	55†
32	No	98.3	1-69	5	—	29 (high)	Strong	86	B	II	80†
33‡	No	100	3-43	0	Weak	5 (low)	—	25	B	II	48†
34‡	No	100	1-69	0	Weak	5 (low)	—	50	C	IV	47
35‡	No	100	3-72	0	Weak	12 (low)	—	2	A	0	87†
36	Yes	97.3	4-61	8	Weak	12 (low)	—	15	B	II	8†
37	Yes	96.6	1-02	10	Weak	4 (low)	—	1	A	0	52†
38	Yes	96.2	4-59	11	Weak	1 (low)	—	0	A	0	138†
39	Yes	95.6	3-07	13	—	11 (low)	—	73	A	0	63†
40	Yes	95.6	4-31	13	—	7 (low)	—	—	A	0	46†
41	Yes	95.6	7-04.1	13	Weak	5 (low)	—	5	A	I	121†
42	Yes	95.6	4-61	13	Weak	2 (low)	—	1	C	IV	289†
43	Yes	93.9	3-21	9	Weak	13 (low)	—	46	A	III	25
44	Yes	93.7	2-05	18	Weak	3 (low)	—	98	A	0	67†
45‡	Yes	93.5	5-51	19	Strong	12 (low)	—	0	A	0	14†
46	Yes	93.3	3-72	20	—	2 (low)	—	78	A	0	64†
47	Yes	93.2	1-03	19	Weak	6 (low)	—	2	A	0	128†
48	Yes	92.5	3-23	22	—	10 (low)	—	—	A	I	59†
49	Yes	92.4	4-34	22	—	1 (low)	—	—	C	IV	9
50	Yes	92.4	4-04	22	—	6 (low)	—	8	A	0	127†
51	Yes	92.4	4-34	22	Weak	6 (low)	—	1	A	0	421†
52	Yes	90.6	4-34	27	Weak	6 (low)	—	12	A	0	122†
53	Yes	89.8	1-18	35	Weak	3 (low)	—	0	A	0	43†
54	Yes	89.1	3-23	32	—	1 (low)	—	—	C	III	56†
55	Yes	89.1	3-74	34	Weak	4 (low)	—	38	A	0	83†
56	Yes	88.4	3-30	34	Weak	4 (low)	—	0	A	0	31†

* Samples in which fewer than 2 percent of base pairs differed from those of the consensus sequence for immunoglobulin heavy-chain variable-region (IgV_H) genes were considered unmutated.⁸ The level of ZAP-70 expression was considered to be high if at least 20 percent of chronic lymphocytic leukemia cells were positive for the protein. Survival was measured from the time of diagnosis. IHC denotes immunohistochemical analysis.

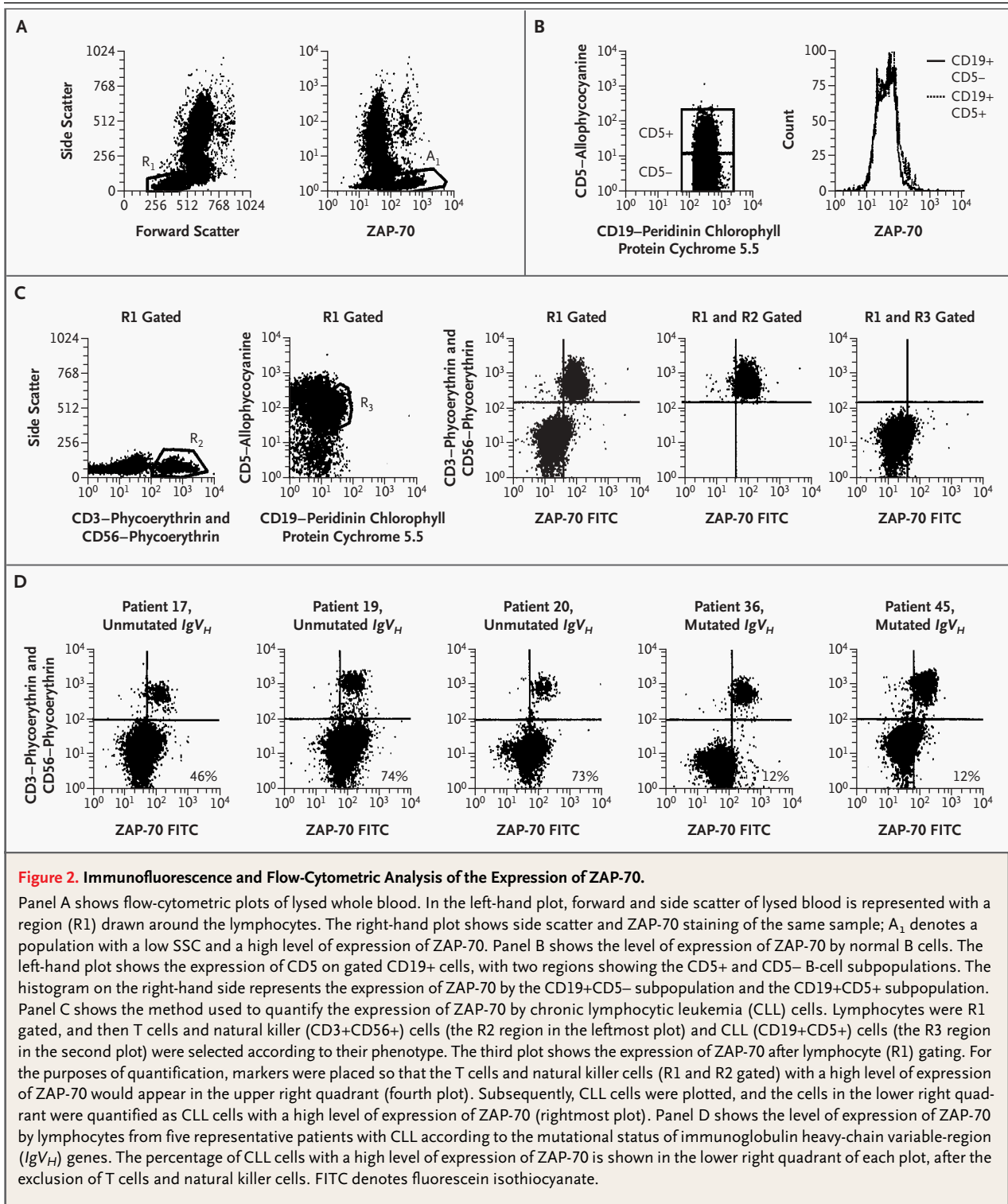
† The patient was still alive at the time of analysis.

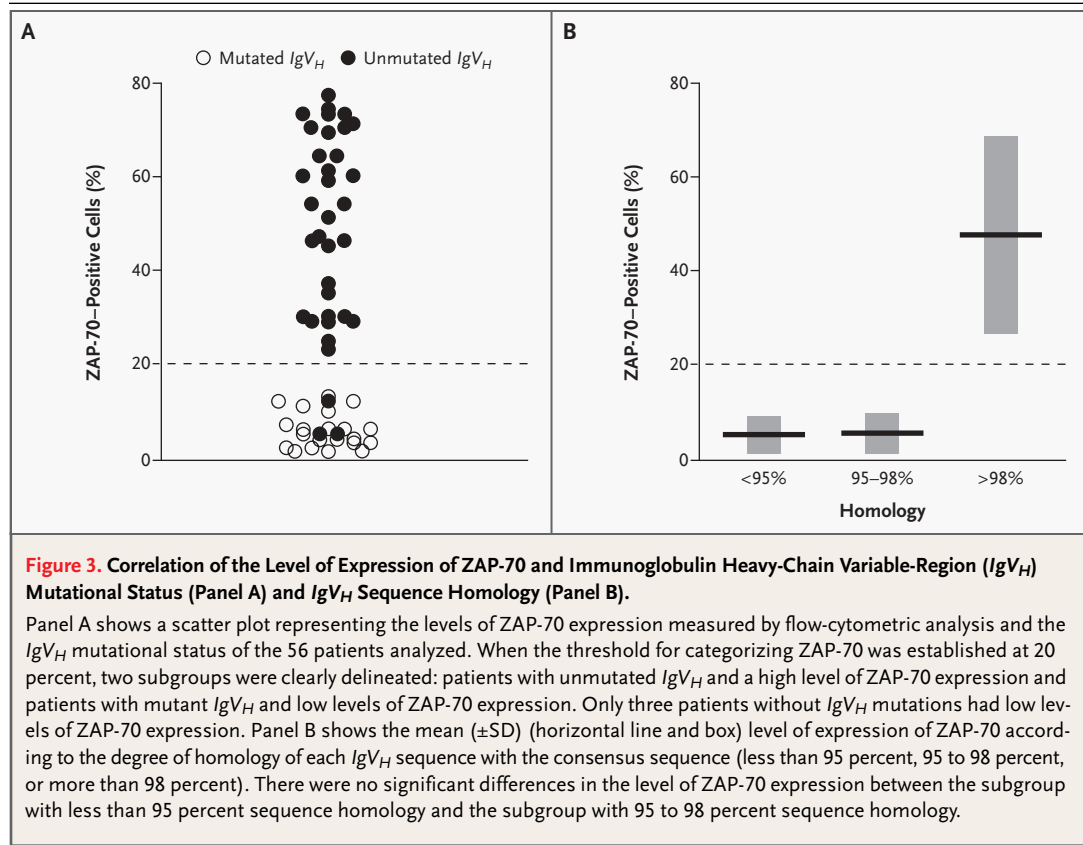
‡ The results of IgV_H mutational analysis and ZAP-70 expression were discordant.



dictive value) was 87.5 percent (95 percent confidence interval, 68 to 97). The sensitivity and specificity of the flow-cytometric analysis were 91 percent (95 percent confidence interval, 77 to 98) and 100 percent (95 percent confidence interval, 84 to 100), respectively.

To verify ZAP-70 expression in the three patients in whom IgV_H status and flow-cytometric results were discordant, Western blot analysis was performed on isolated fractions of CD19+ cells with a purity greater than 98 percent (CD19 is a surface marker of B cells). A weak signal was observed in all





three samples (data not shown), validating the low levels of expression of ZAP-70 observed on flow cytometry.

ZAP-70 AND CD38 EXPRESSION

Data for the expression of CD38 were available for 45 patients (CD38 is a marker that has been proposed as a surrogate for *IgV_H* mutational status). Of these 45 patients, 18 had no more than 30 percent CD38+ cells (Table 1). There were significant differences in the mean percentages of CD38+ CLL cells between patients with *IgV_H* mutations and those without *IgV_H* mutations (20 percent vs. 60 percent, $P < 0.001$). In addition, 22 of 28 patients without *IgV_H* mutations (79 percent) had at least 30 percent CD38+ cells, whereas 12 of 17 (71 percent) with somatic mutations had less than 30 percent CD38+ cells. Moreover, the percentage of CD38+ cells was at least 30 percent in 21 of 25 patients (84 percent) with at least 20 percent ZAP-70-positive cells and less than 30 percent in 14 of 20 patients (70 percent) with less than 20 percent ZAP-70-positive cells. The level of CD38 expression was also low in the two pa-

tients with discordant results of ZAP-70 expression and *IgV_H* mutations (Table 1). In a multiple regression analysis, only ZAP-70 expression ($P < 0.001$), and not CD38 expression, maintained its correlation with *IgV_H* mutational status.

PROGNOSTIC IMPORTANCE OF THE PERCENTAGE OF ZAP-70-POSITIVE CELLS

None of the standard variables were associated with ZAP-70 expression, including Binet's and Rai's clinical stages, the lymphocyte count, and the lymphocyte doubling time. However, an increased percentage of ZAP-70-positive cells was associated with a short time to progression. Among 44 patients with Binet stage A, the median time to progression was 29 months for the 26 patients with at least 20 percent ZAP-70-positive cells, whereas the median was not reached among the 18 patients with less than 20 percent ZAP-70-positive cells ($P = 0.009$) (Fig. 4A).

When survival was calculated from the time of diagnosis, the 26 patients with Binet stage A CLL and at least 20 percent ZAP-70-positive cells had a median survival of 90 months, whereas the median sur-

vival was not reached in the 18 patients with Binet stage A and less than 20 percent ZAP-70-positive cells ($P=0.01$) (Fig. 4B). However, when all patients were analyzed (Binet stage A, B, and C), the percentage of ZAP-70-positive cells was not significantly correlated with survival ($P=0.06$). The percentage of CD38+ cells (with a cutoff of 30 percent) did not predict progression or survival (data not shown). Finally, as expected, IgV_H mutational status also correlated with progression and survival (data not shown).

DISCUSSION

The management of CLL is based on each patient's individual risk, because the disease has a widely variable clinical course. An important determinant of the prognosis is the mutational status of IgV_H genes in the leukemic cells, which correlates with the clinical outcome better than and independently of classic prognostic factors.^{8,11,31} Methods to identify IgV_H mutations are, however, not widely available in clinical practice. Our study confirms that the leukemic cells without IgV_H mutations in patients with CLL express high levels of ZAP-70, whereas the leukemic cells with IgV_H mutations in patients with CLL have barely detectable levels of ZAP-70.^{16,17} Moreover, using flow cytometry, we found that none of the patients with CLL and at least 20 percent ZAP-70-positive cells had IgV_H somatic mutations, whereas all but three patients with less than 20 percent ZAP-70-positive cells had mutated IgV_H genes. Flow-cytometric analysis of ZAP-70 expression was a sensitive and specific surrogate for mutational status of IgV_H genes; this method should be readily available in general laboratories.

The mechanisms accounting for the relation between ZAP-70 expression and the mutational status of IgV_H are unknown. We also have no explanation for the inconsistency between mutational status and ZAP-70 levels in three of our patients. A discrepancy between ZAP-70 expression, as assessed by Western blotting, and the IgV_H mutational status was also found in 1 of 22 patients in another series.¹⁷

Apart from CLL cells, ZAP-70 was expressed only in T cells and tumors of T-cell lineage, whereas it was barely detectable in normal CD19+CD5+ cells. It was not detected by Western blotting or immunohistochemistry in any of the B-cell lines or biopsy specimens of mantle-cell lymphoma. Nevertheless, ZAP-70 messenger RNA has been found in other B-cell lines with the use of reverse-transcriptase-

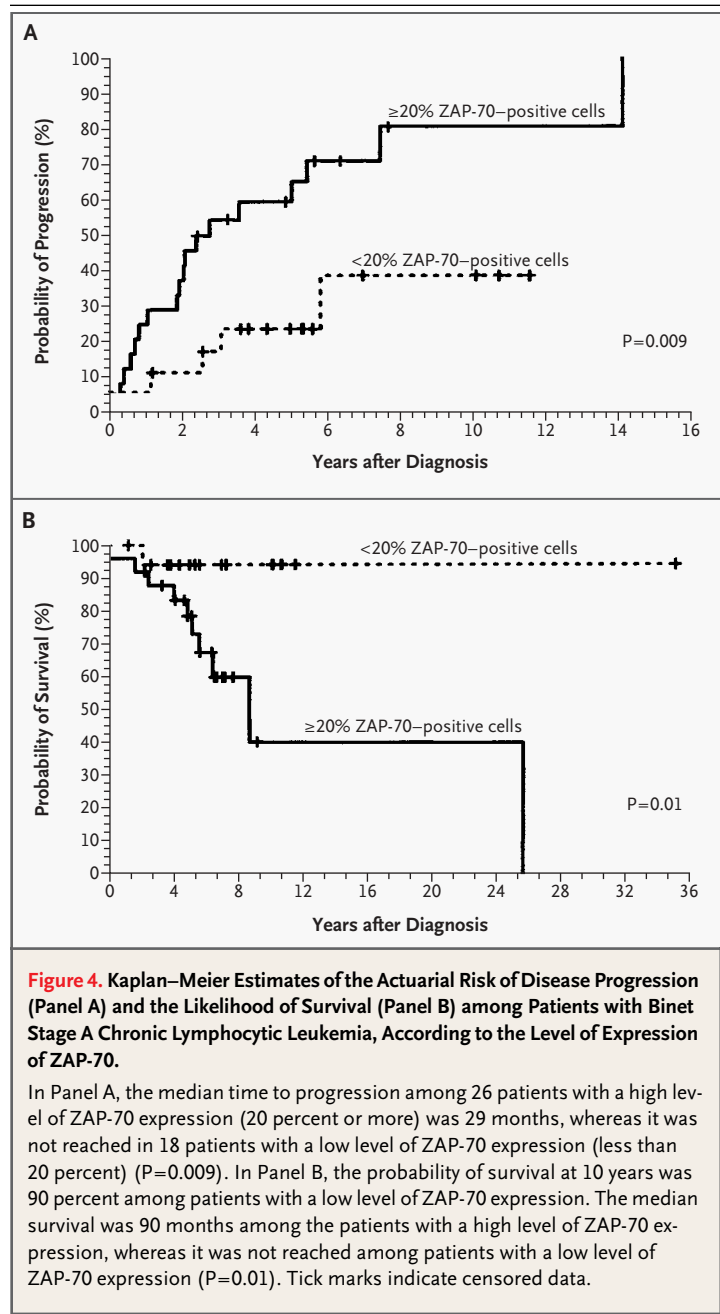


Figure 4. Kaplan-Meier Estimates of the Actuarial Risk of Disease Progression (Panel A) and the Likelihood of Survival (Panel B) among Patients with Binet Stage A Chronic Lymphocytic Leukemia, According to the Level of Expression of ZAP-70.

In Panel A, the median time to progression among 26 patients with a high level of ZAP-70 expression (20 percent or more) was 29 months, whereas it was not reached in 18 patients with a low level of ZAP-70 expression (less than 20 percent) ($P=0.009$). In Panel B, the probability of survival at 10 years was 90 percent among patients with a low level of ZAP-70 expression. The median survival was 90 months among the patients with a high level of ZAP-70 expression, whereas it was not reached among patients with a low level of ZAP-70 expression ($P=0.01$). Tick marks indicate censored data.

PCR (RT-PCR) analysis.¹⁶ Our results indicate that, among B-cell and T-cell lymphoproliferative disorders, a high level of ZAP-70 expression is restricted to T-cell proliferative diseases and a subgroup of CLL.

The use of flow cytometry to assess the level of ZAP-70 expression offers advantages over other systems of analysis. With flow cytometry, it is possible

to analyze the percentage of ZAP-70–positive cells selectively in subpopulations of CLL cells, T cells, and natural killer cells. By contrast, Western blotting and RT-PCR can overestimate the level of expression of ZAP-70 owing to the presence of T cells in the sample.^{16,17}

The value of CD38 as a surrogate for IgV_H mutations is controversial.^{32,33} Moreover, the level of CD38 expression may vary over the course of the disease.³⁴ In our series, the level of CD38 expression did not correlate with disease progression or survival. In contrast, the level of expression of ZAP-70 did not change over time, and the presence of ZAP-70 was associated with rapid progression and poor survival.

In conclusion, the expression of ZAP-70 by CLL cells, as ascertained by flow-cytometric analysis, is a simple and reliable surrogate for the identification of IgV_H mutations. Moreover, ZAP-70 expression by itself can be used as a prognostic marker. For these reasons, ZAP-70 analysis should be included in the workup of patients with CLL.

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CORRECTION**ZAP-70 in Chronic Lymphocytic Leukemia**

To the Editor: Crespo et al. (May 1 issue)¹ present informative data on ZAP-70 as a surrogate prognostic marker for chronic lymphocytic leukemia (CLL). The patients in their study had heterogeneous backgrounds and may have been treated by different treatment strategies according to their age and disease status. Elderly patients with advanced or refractory CLL may have received less intensive treatment than others, leading to a poorer prognosis. Because treatments differ greatly in their effects on overall survival, there may have been a selection bias in the study. The authors have not provided sufficient information on these variables. Clinicians will appreciate it if the authors can share the data on treatments.

Naoko Murashige, M.D.
Masahiro Kami, M.D.
Yoichi Takaue, M.D.
National Cancer Center Hospital
Tokyo 104-0045, Japan
mkami@ncc.go.jp

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1. Crespo M, Bosch F, Villamor N, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med* 2003;348:1764-1775.

The authors reply: Murashige et al. make interesting comments concerning the clinical characteristics and treatment of the patients in our study. At our institution, patients in whom Binet stage A CLL is diagnosed do not receive therapy unless they meet criteria for active CLL.¹ Treatment has, of course, varied over the years, from chlorambucil to fludarabine-based combination regimens. Interestingly, in our series, only 3 of the 18 patients with Binet stage A disease and low ZAP-70 expression required treatment, whereas 20 of the 26 patients with high ZAP-70 expression were treated. The main clinical variables, including age, did not differ between the two groups. Thus, with ZAP-70 analysis we were able to identify a group of patients with a bad prognosis, irrespective of the clinical stage. Finally, the relation among ZAP-70 expression, clinical variables, and survival should be assessed in larger series of patients.

The source of the monoclonal anti-ZAP-70 antibody used for flow cytometry was Upstate Biotechnology.

Francesc Bosch, M.D.
Neus Villamor, M.D.
Emili Montserrat, M.D.
Hospital Clinic
Barcelona 08036, Spain
fbosch@clinic.ub.es

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