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ORIGINAL REPORT

Additional Genetic High-Risk Features Such As 11q Deletion, 17p Deletion, and *V3-21* Usage Characterize Discordance of ZAP-70 and *VH* Mutation Status in Chronic Lymphocytic Leukemia

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ABSTRACT

Purpose
Immunoglobulin heavy chain variable-region (*VH*) gene mutation status and zeta-associated protein 70 (ZAP-70) expression are correlated in chronic lymphocytic leukemia (CLL), but their concordance is variable. The goal of this study was to elucidate additional factors potentially characterizing their discordance.

Patients and Methods
We evaluated ZAP-70 expression by flow cytometry, *VH* status by DNA sequencing, and genomic aberrations by fluorescence in situ hybridization in 148 CLL patients. The parameters were analyzed for their associations and their individual prognostic impact.

Results
ZAP-70 expression and *VH* mutation status were strongly associated in CLL without additional genetic high-risk-features as defined by the absence of 11q or 17p deletion and *V3-21* usage (concordance 84%). In contrast, the proportion of discordant cases was significantly higher (39%), if such additional genetic high-risk features were present. Discordant cases with *V3-21* usage were almost exclusively ZAP-70 positive and *VH* mutated (89%), whereas all but one of the discordant cases with high-risk aberrations were ZAP-70 negative and *VH* unmutated (92%). By multivariate regression analysis, two models were developed, which both include high-risk genomic aberrations and, alternatively, *VH* mutation status and *V3-21* usage or ZAP-70 expression as independent outcome predictors.

Conclusion
There were characteristic modes of discordance between ZAP-70 and *VH* mutation status depending on the presence or absence of additional genetic high-risk features such as 11q and 17p deletion or *V3-21* usage. Although the biologic background for these findings is yet to be determined, these data have biologic and clinical implications regarding ZAP-70 as a pathogenic factor and outcome predictor, respectively.

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INTRODUCTION

The clinical course of chronic lymphocytic leukemia (CLL) is highly variable with survival times ranging from months to decades.[1-3] The clinical staging systems according to Rai[4] and Binet[5] are of prognostic importance, but they cannot predict the outcome of individual patients in early clinical stages. To develop risk adapted treatment strategies, prognostic factors are needed, which allow the prediction of the individual patients clinical course.[6]

The *VH* mutation status and genomic aberrations have been shown to be independent prognostic factors in CLL.[7-11] In addition, the rearrangement of a specific variable-region (*VH*) gene, the *V3-21* gene, has been associated with an unfavorable clinical outcome irrespective of the *VH* mutation status.[12-14,14A] Therefore, we have considered *V3-21* using cases as a separate risk group independent of the *VH* mutation status.

Zeta-associated protein 70 (ZAP-70) is physiologically involved in the signal transduction of the T-cell receptor (TCR).[15,16] Normal "B" lymphocytes do not express ZAP-70, but microarray analyses have shown that ZAP-70 is overexpressed in *VH* unmutated CLL and could therefore serve as a

surrogate marker for the *VH* mutation status.[17,18] In vitro studies demonstrated that ZAP-70 is involved in the signal transduction cascade initiated by B cell receptor (BCR) stimulation in *VH* unmutated CLL, implicating a functional role in pathogenesis and clinical behavior.[19,20,3] Most importantly, a strong correlation between ZAP-70 expression and the clinical course of CLL has been reported, but ZAP-70 and *VH* mutation status showed a variable degree of concordance in different studies ranging from 77% to 95%.[17,21-26] The causes for these interstudy differences are not clear and it is not known whether discordance of ZAP-70 and *VH* status may be characterized by specific patterns of additional genetic features of independent prognostic impact in CLL.

To address these questions, we analyzed ZAP-70 expression, the *VH* mutation status, and other potentially independent biologic prognostic factors such as *V3-21* usage and genomic aberrations in a large cohort of CLL patients.

PATIENTS AND METHODS

Patient Cohort

In the current study, 148 CLL patients observed at our institution were enrolled after informed consent. ZAP-70 flow cytometry was performed in all enrolled cases. *VH* sequencing and fluorescence in situ hybridization (FISH) analysis were performed in all cases where suitable samples were available (133 and 144 cases, respectively). Clinical data were available for 139 cases. The median age at diagnosis was 57 (range, 25 to 79). The male to female ratio was 1.84. At the time of diagnosis, the distribution of Binet stages A, B, and C was 65%, 27%, and 8%, respectively. The Binet stage at diagnosis was unknown in 16 cases. The median times from diagnosis to ZAP-70, *VH*, and FISH analyses were 28, 26, and 26 months, ranging from 0 to 260 months, respectively. At the time of ZAP-70 analysis, 72% of patients were untreated. Eighty-six patients required therapy and 23 deaths occurred within a median follow-up time of 53 months.

ZAP-70 Expression Analysis by Flow Cytometry

ZAP-70 expression was measured by four-color flow cytometry (CD5, CD19, CD3/56, ZAP-70) according to Crespo et al.[21] Based on availability, ZAP-70 was measured either in fresh blood samples or in mononuclear cells from frozen samples. The cells were fixed, permeabilized (IntraPrep, Beckman Coulter, Krefeld, Germany), and stained with the anti–ZAP-70 antibody (clone 2F3.2, Upstate; Biomol, Hamburg, Germany). After washing with phosphate-buffered saline (PBS) –bovine serum albumin 0.1%, goat antimouse-FITC (Dako, Hamburg, Germany) was added. After washing and blocking, the cells were stained with anti-CD3-PE (clone SK7, BD Bioscience, Heidelberg, Germany), anti-CD56-PE (clone My 31, BD Bioscience), anti-CD19-ECD (clone HD237, Beckman Coulter) and anti-CD5-PC5 (clone BL1a, Beckman Coulter). The samples were washed and subsequently analyzed by flow cytometry (EPICS XL-MCL, Beckman Coulter).

Analysis of Genomic Aberrations and VH Status

FISH and *VH*-gene sequencing were performed as previously described.[7,10] The chromosomal regions studied were 6q21, 11q13, 11q22-q23, 12q13, 13q14, 17p13, and 14q32. A sequence homology cutoff of 98% was used to define the *VH* mutation status.

Statistical Analysis

The primary end points were treatment-free survival (TFS) and overall survival (OS) from time of diagnosis. Differences of OS and TFS distributions were analyzed by log-rank statistics. Survival curves were plotted using Kaplan-Meier estimates. The median duration of follow-up was calculated according to the Korn method.[27] The Cox proportional hazards regression model was used to identify differences in TFS distributions between subgroups.[28] As possible prognostic factors, the presence of high-risk genomic aberrations (17p and 11q deletion), *V3-21* usage, unmutated *VH* status in the absence *V3-21* usage, and ZAP-70 expression in $\geq$ 20% of CLL cells were included. Missing data were estimated with a multiple-imputation technique using predictive mean matching with n = 100 imputations.[29] Categoric values were analyzed with the Fisher's exact test. All tests were two sided. An effect was considered significant at $P < .05$. To provide quantitative information on the relevance of the results, the 95% CI values were computed. Statistical analyses were performed with R[30] and with GraphPad Prism version 3.00 (GraphPad Software, San Diego, CA).

RESULTS

Association Between ZAP-70 Expression, VH Mutation Status, and Additional Genetic High-Risk-features Such As 11q or 17p Deletion and V3-21 Usage

Sixty-five of 148 (44%) cases showed ZAP-70 expression in less than 20% of cells, and were considered ZAP-70 negative, whereas 83 of 148 (56%) cases exhibited ZAP-70 expression in $\geq$ 20% of cells, and were considered ZAP-70 positive. *VH* mutation analysis was available for 133 cases. *VH* genes were mutated in 53 of 133 (40%) cases and unmutated in 80 of 133 (60%) cases. The *V3-21* gene was clonally rearranged in 16 of 133 (12%) cases and was mutated in 11 of 16 (69%) cases. Genomic aberrations were detected in 104 of 144 (72%) cases. 13q deletion was present in 75 of 144 (52%), 13q deletion as a single aberration in 48 of 144 (33%) cases, 11q deletion in 30 of 144 (21%) cases, 17p deletion in 17 of 144 (12%) of cases, trisomy 12 in 12 of 144 (8%) cases, 14q involving aberrations in 6 of 144 (4%), and 6q deletion in 2 of 144 (1%).

Concordance of ZAP-70 expression and *VH* mutation status was observed in 100 of 133 (75%) cases (ZAP-70 negative/*VH* mutated: 38 of 53 (72%); ZAP-70 positive/*VH* unmutated: 62 of 80 [78%]). Discordance was observed in 33 of 133 (25%) cases (ZAP-70 negative/*VH* unmutated: 18 of 80 (23%); ZAP-70 positive/*VH* mutated: 15 of 53 [28%]). When comparing the association of ZAP-70 expression and *VH* mutation status in subgroups defined by the presence or absence of additional genetic high-risk features such as 17p deletion, 11q deletion, or *V3-21* usage, striking differences were observed (Fig 1, Table 1, and Table 2). The association between ZAP-70 expression and *VH* mutation status was significantly stronger in the subgroup without additional genetic high-risk features as compared to the group with additional genetic high-risk features (concordance: 65 of 76 (86%) *v* 34 of 56 (61%); $P = .002$). Compared to the *VH* mutated/ZAP-70 negative subgroup high-risk genomic aberrations were significantly more frequently observed in the *VH* unmutated/ZAP-70 positive subgroup (24 of 27 (89%); $P < .001$; Table 1). Furthermore, the distribution of the two distinct discordance modes—ZAP-70 negative/*VH* unmutated and ZAP-70 positive/*VH* mutated—were strikingly different when comparing individual genetic high-risk categories (Table 1 and Table 2). Discordant cases with 11q or 17p deletion, but no *V3-21* usage were almost exclusively ZAP-70 negative/*VH* unmutated (12 of 13 [92%], $P < .001$), whereas discordant cases with a *V3-21* rearrangement were almost exclusively ZAP-70 positive/*VH* mutated (8 of 9 [89%]; $P = .004$). In contrast, a balanced distribution of the discordance modes was observed among cases without 11q or 17p deletion and no *V3-21* usage (ZAP-70 negative/*VH* unmutated *v* ZAP-70 positive/*VH* mutated: 5 of 11 [45%] *v* 6 of 11 [55%]).

Prognostic Impact of ZAP-70 Expression and VH Status in Univariate Analyses

The median OS and TFS of the ZAP-70 positive subgroup was significantly shorter compared with the ZAP-70 negative subgroup

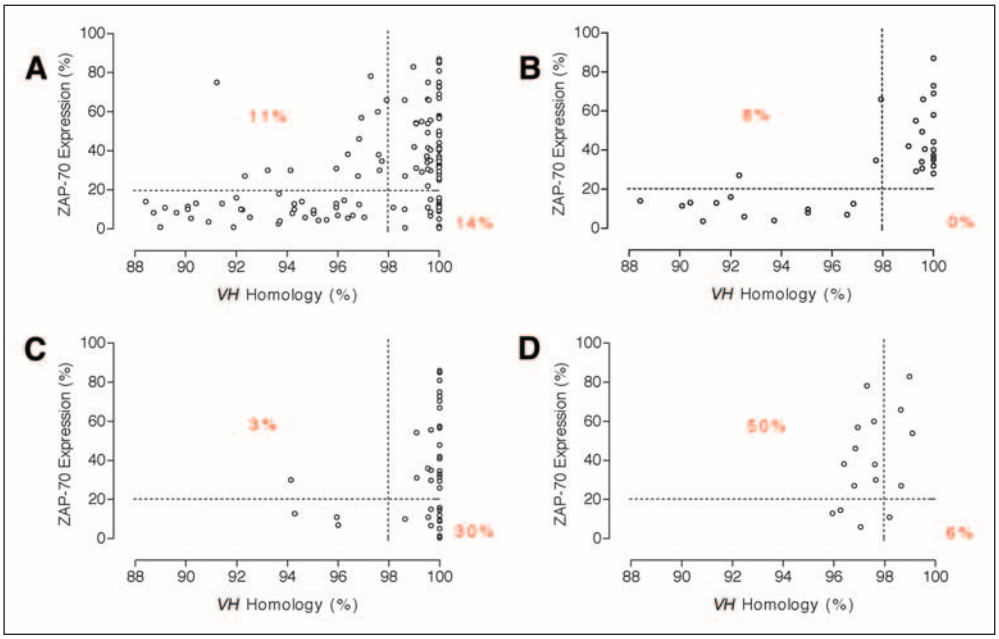

Fig 1. Association of zeta-associated protein 70 (ZAP-70) expression and the variable-region gene (*VH*) mutation status in chronic lymphocytic leukemia (CLL) subgroups defined by the presence or absence of additional genetic high-risk features, such as 11q or 17p deletion and *V3-21* usage. (A) All cases (n = 133); (B) normal karyotype, no *V3-21* usage (n = 36); (C) 11q or 17p deletion, no *V3-21* usage (n = 40); (D) *V3-21* usage (n = 16). The horizontal dotted line represents the ZAP-70 cutoff value of 20%. The vertical dotted line represents the *VH* homology cutoff value of 98%.

(median OS, 100 months *v* last observed death at 76 months; survival probability, 85%; $P = .004$; median TFS, 24 *v* 50 months; $P = .005$; Fig 2).

For survival analyses according to the *VH* status, the *V3-21* exhibiting cases were considered as a mutation status independent prognostic subgroup and the cohort was therefore divided into three groups: (1) *V3-21*—cases with a *V3-21* rearrangement irrespective of the mutation status; (2) *VH* unmutated—cases with an unmutated *VH*, but no *V3-21* rearrangement; and (3) *VH* mutated—cases with a mutated *VH*, but no *V3-21* rearrangement. The median OS and TFS in the groups *V3-21* (n = 15) and *VH* unmutated (n = 70) were significantly shorter compared to the *VH* mutated group (n = 40; median OS: 94 *v* 111 months *v* last observed death at 15 months; survival probability 96%; $P = .013$; median TFS, 22 *v* 24 *v* 172 months; $P < .001$; Fig 2).

Survival data were available for 31 of 33 cases with discordant ZAP-70 and *VH* mutation status (Table 2). Among the discordant cases without additional genetic high-risk features, the clinical course was as expected for the individual *VH* mutation status and not for the ZAP-70 expression in all cases. Among the discordant cases with presence of additional genetic high-risk features, a characteristic pattern was observed: the subgroup with *V3-21* usage showed almost exclusively a high ZAP-70 expression despite a mutated *VH* status and an aggressive clinical course. In contrast, the subgroup with 11q or 17p deletion showed in almost all cases a low ZAP-70 expression despite an unmutated *VH* status and an aggressive clinical course (Table 2).

Prognostic Impact of ZAP-70 Expression, VH *Mutation Status,* V3-21 *Usage, and High-Risk Genomic Aberrations in Multivariate Analyses*

ZAP-70 expression, *VH* mutation status, *V3-21* usage, and high-risk genomic aberrations were analyzed for their independent prognostic value applying the proportional hazards regression model of Cox. When all factors were included in the model, unmutated *VH*, *V3-21* usage, and high-risk genomic aberrations, but not ZAP-70 expression, were identified as independent predictors for TFS. Alternatively, in a model excluding the *VH* mutation status and *V3-21* usage, ZAP-70 expression was selected as a significant predictor in addition to the high-risk genomic aberrations 11q and 17p deletion (Table 3). Due to small event numbers in the

Table 1. Distribution of Cases According to the *VH*/ZAP-70 Status and According to the Presence or Absence of Additional Genetic High-Risk Features

	Concordant Cases						Discordant Cases					
		VH Mutated/ ZAP-70 Negative		*VH* Unmutated/ ZAP-70 Positive				*VH* Mutated/ ZAP-70 Positive		*VH* Unmutated/ ZAP-70 Negative		
Genetic Characterization	No. of Patients	No.	%	No.	%	*P**	No. of Patients	No.	%	No.	%	*P**
17p- or 11q- (no *V3-21*)	27	3	11	24	89	.001	13	1	8	12	92	.001
V3-21	7	3	43	4	57	NS	9	8	89	1	11	.004
Other	65	32	49	33	51	NS	11	6	55	5	45	NS

Abbreviations: *VH*, variable region; ZAP-70, zeta-associated protein 70; NS, not significant.
*Fisher's exact test.

Table 2. Genetic Characterization and Clinical Course of Cases With Discordant ZAP-70 and *VH* Mutation Status

ZAP-70–Positive Cells (%)	*VH* Homology (%)	*V3-21*	11q	17p	Treatment-Free Survival (months)	Therapy	Initial Therapy Before FISH	Initial Therapy Before ZAP-70 FC	Binet Stage at Diagnosis
No additional genetic high risk features									
75	91.23	No	No	No	52	No	No	No	A
27	92.34	No	No	No	64	No	No	No	A
30	93.24	No	No	No	10	No	No	No	A
31	95.95	No	No	No	50	No	No	No	A
35	97.74	No	No	No	25	No	No	No	A
66	97.93	No	No	No	61	No	No	No	A
1	**98.65**	No	No	No	**90**	**Yes**	**Yes**	**Yes**	**A**
9	**99.66**	No	No	No	**0**	**Yes**	**No**	**No**	**C**
13	**100**	No	No	No	**6**	**Yes**	**No**	**No**	**A**
11	**100**	No	No	No	**104**	**Yes**	**Yes**	**Yes**	**B**
12	**100**	No	No	No	**58**	**Yes**	**No**	**No**	**A**
Additional genetic high risk features									
38	96.40	**Yes**	**Yes**	No	**28**	**Yes**	**No**	**No**	**C**
27	96.80	**Yes**	No	No	36	No	No	No	A
46	96.85	**Yes**	**Yes**	No	14	No	No	No	B
57	96.94	**Yes**	**Yes**	No	**8**	**Yes**	**No**	**No**	**A**
78	97.30	**Yes**	No	No	**22**	**Yes**	**No**	**No**	**A**
60	97.58	**Yes**	No	No	**1**	**Yes**	**Yes**	**Yes**	**B**
38	97.62	**Yes**	No	No	36	No	No	No	B
30	94.14	No	No	**Yes**	**0**	**Yes**	**No**	**No**	**B**
11	**98.20**	**Yes**	No	No	**17**	**Yes**	**No**	**No**	**B**
10	**98.64**	No	**Yes**	No	**13**	**Yes**	**Yes**	**Yes**	**B**
12	**100**	No	**Yes**	No	**27**	**Yes**	**No**	**No**	**Unknown**
1	**100**	No	**Yes**	No	**87**	**Yes**	**No**	**No**	**A**
15	**100**	No	**Yes**	No	**48**	**Yes**	**No**	**No**	**A**
5	**100**	No	**Yes**	No	**92**	**Yes**	**No**	**Yes**	**A**
1	**100**	No	**Yes**	No	**7**	**Yes**	**No**	**No**	**Unknown**
9	**100**	No	**Yes**	No	**62**	**Yes**	**No**	**No**	**C**
11	**99.55**	No	No	**Yes**	**29**	**Yes**	**Yes**	**Yes**	**A**
15	**99.66**	No	No	**Yes**	**0**	**Yes**	**Yes**	**Yes**	**Unknown**
10	**100**	No	No	**Yes**	**37**	**Yes**	**Yes**	**Yes**	**A**
16	**100**	No	No	**Yes**	**1**	**Yes**	**No**	**No**	**B**

NOTE. Risk factor positive or unfavorable clinical course are boldfaced.
Abbreviations: ZAP-70, zeta-associated protein 70; *VH,* variable region; FISH, fluorescent in situ hybridization; FC, flow cytometry.

individual risk groups, multivariate analyses were not performed for OS.

Based on the results of the Cox regression analyses, two prognostic models were developed (Fig 3). The first model was composed of five genetic subgroups, in which each patient was allocated to one category only: (1) 17p deletion, no *V3-21* usage; (2) 11q deletion, no 17p deletion, and no *V3-21* usage; (3) *V3-21* usage; (4) *VH* unmutated without additional genetic high-risk features; and (5) *VH* mutated without additional genetic high-risk features. The median TFS for these categories was 15 months for 17p deletion, 20 months for 11q deletion, 22 months for *V3-21* usage, 31 months for the *VH* unmutated group, and 172 months for the *VH* mutated group. In the second model, *VH* mutation status and *V3-21* usage were replaced by the ZAP-70 status. The following four groups, in which each patient was allocated to one category only, were defined: (1) 17p deletion irrespective of the ZAP-70 status; (2) 11q deletion irrespective of the ZAP-70 status; (3) ZAP-70 positive, no high-risk genomic aberrations; and (4) ZAP-70 negative, no high-risk genomic aberrations. The median TFS for these categories was 15 months for 17p deletion, 20 months for 11q deletion, 31 months for the ZAP-70 positive group, and 86 months for the ZAP-70 negative group. The prognostic impact of the categories defined in the two models was retained when analyzing OS (Fig 3).

DISCUSSION

In this study, we analyzed the association and the prognostic impact of ZAP-70 expression, the *VH* mutation status, and additional independent genetic high-risk factors such as *V3-21* usage and genomic aberrations in CLL. In agreement with previous reports, a strong association of high ZAP-70 expression and unmutated *VH*-genes was confirmed.[17,21,23-25] However, discordance of ZAP-70 expression and *VH* mutation status occurred in 25% of cases. In previously published studies, the proportion of discordant cases ranged from 5% to 23%.[21,23,24] These differences may be related to different flow cytometric detection strategies, (ie, the use of various antibody clones, unlabeled or directly fluorochrome labeled antibodies, and diverse gating strategies.) However, the methodology was successfully validated by additional approaches (ie, immunoblotting, cDNA microarray).[21,23,24] Our data implicate that the majority of ZAP-70 and *VH*

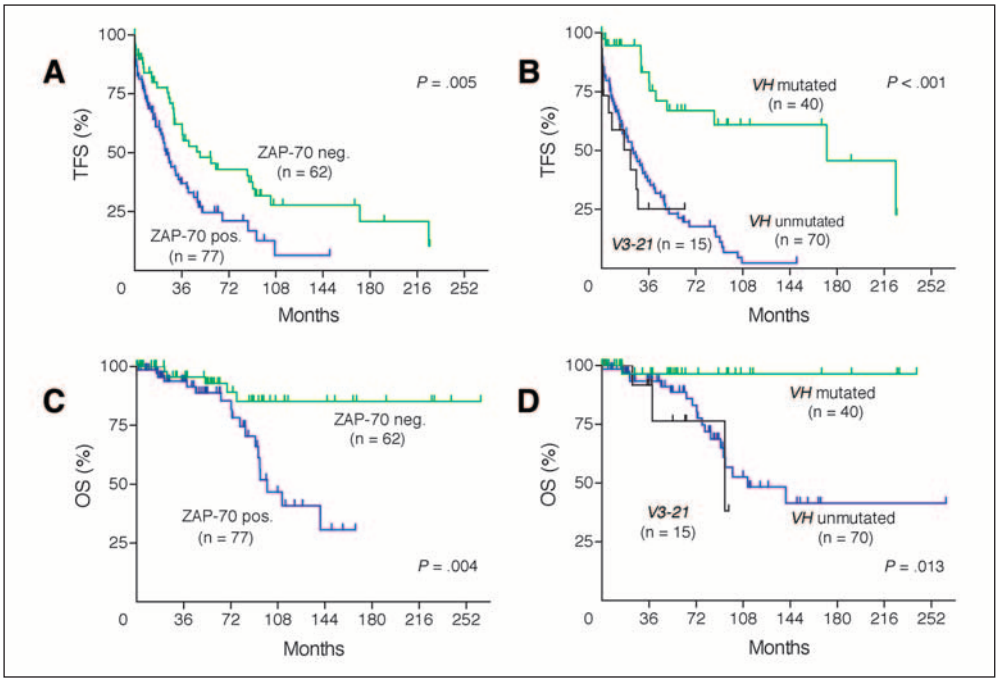

Fig 2. Kaplan-Meier curves of treatment-free survival (TFS; A and B) and overall survival (OS; C and D) according to zeta-associated protein 70 (ZAP-70) expression (A and C) and the variable-region gene (*VH*) status (B and D) are shown. pos., positive; neg., negative.

mutation status discordant cases may be explained by high rates of discrepancies in the presence of additional genetic high-risk features. The composition of the patient cohorts of published studies supports this concept. Crespo et al[21] and Orchard et al[23] who found high concordance rates (95% and 92%, respectively) analyzed cohorts with high proportions of Binet A patients (79% and 86%, respectively). Furthermore, in the study of Orchard et al,[23] 68% of cases were *VH* mutated, and the incidence of high-risk genomic aberrations was low (17p deletion, 4%; 11q deletion, 8%). In contrast, Rassenti et al,[24] who found a strikingly lower concordance proportion (77%), analyzed a patient cohort frequently exhibiting biologic high-risk features (53% *VH* unmutated). Their cohort was derived from referral centers mainly enrolling clinically advanced patients, who were treated in approximately half of the cases.

The data presented in this article indicate alternative, biologic mechanisms as a basis for the discordances. The association between ZAP-70 expression and the *VH* mutations status differed significantly according to presence or absence of additional genetic high-risk features. The proportion of discordant cases was particularly high in the subgroups with *V3-21* usage and 17p or 11q deletion (39%). Interestingly, the mode of the discordances (ZAP-70 positive/*VH* mutated or ZAP-70 negative/*VH* unmutated) within these genetic high-risk categories showed striking differences. Cases with *V3-21* usage were almost exclusively ZAP-70 positive/*VH* mutated (89%), whereas all but one of the discordant cases with high-risk genomic aberrations were ZAP-70 negative/*VH* unmutated (92%). In contrast, the association of ZAP-70 expression and the *VH* mutation status was stronger in cases without *V3-21* usage, 17p deletion, or 11q deletion (84% concordance), and the distribution of the discordance categories was balanced (ZAP-70 positive/*VH* mutated *v* ZAP-70 negative/*VH* unmutated cases, 55% *v* 45%).

The current data provide evidence for a recently proposed theoretical model of ZAP-70 expression in the pathogenesis of CLL, in which active BCR signaling stimulates the malignant clone.[3] A survival advantage for CLL cells as a result of the BCR related interaction with unknown antigens could explain the signs of antigen selection pressure, such as restricted *VH*-gene usage, particularly in the *VH* unmutated subgroup, restricted VHDJH-combinations (immunoglobulin heavy chain variable [VH], diversity [DH], and joining [JH] gene segments),[14,31,32] and the occurrence of highly homologous *VH*-genes involving BCRs with nearly identical complementarity determining region 3 regions,[12,13,33,34] a conserved *V3-21* structure reflected by a remarkably narrow mutation range (mainly between 2% and 4%, compare Fig 1D), restricted *JH6* usage, and a restricted usage the

Table 3. Multivariate Regression Analyses of ZAP-70 Status, *VH* Status, and Genomic Aberrations According to Treatment-Free Survival in CLL

Parameter	Hazard Ratio	95% CI	*P*	Hazard Ratio	95% CI	*P*
VH unmutated	2.9	1.6 to 5.6	< .001		—	—
V3-21 usage	2.6	1.1 to 6.3	.035		—	—
High-risk genomic aberrations	1.7	1.1 to 2.8	.027	2.2	1.4 to 3.4	< .001
ZAP-70 positive	1.2	0.7 to 2.0	.48*	1.9	1.2 to 3.9	.006†

Abbreviations: ZAP-70, zeta-associated protein 70; *VH,* variable region; CLL, chronic lymphocytic leukemia.
*ZAP-70 was not selected as a significant prognostic parameter if the *VH* status was included in the multivariate analysis.
†ZAP-70 was selected as a significant prognostic parameter if the *VH* status was not included in the multivariate analysis.

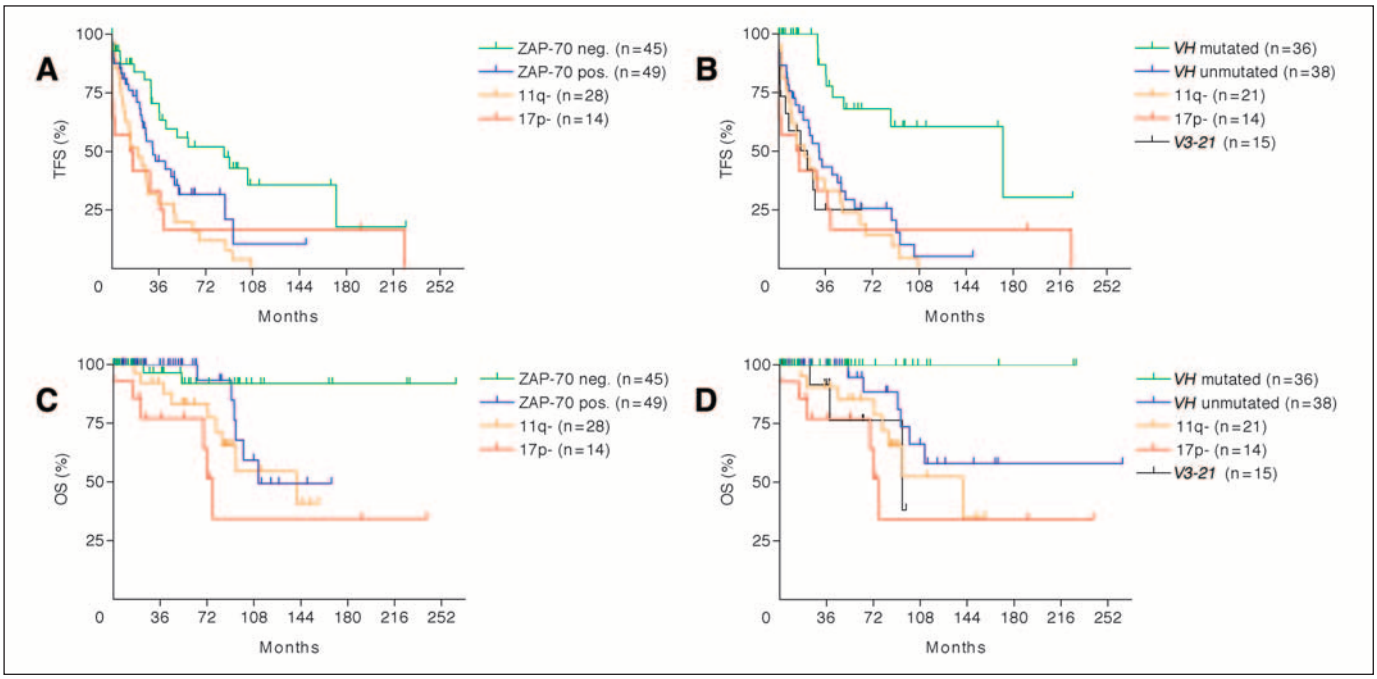

Fig 3. Kaplan-Meier curves of treatment-free survival (TFS; A and B) and overall survival (OS; C and D) are shown. Based on multivariate regression analyses, two risk models were developed (compare text for details). Both models included high-risk genomic aberrations. In addition, zeta-associated protein 70 (ZAP-70) expression (A and C) or the variable-region gene (*VH*) status (B and D) were alternatively included. pos., positive; neg., negative.

Vλ2-14-light chain gene in *V3-21* rearrangements.[12,13] ZAP-70 has been shown in vitro to be involved in the BCR signal transduction in CLL cells.[19,20] In the majority of *VH* unmutated cases and, most interestingly, in the majority of cases with a mutated *V3-21* gene, ZAP-70 was upregulated in our study pointing to active BCR signaling.[30A] However, we observed a high rate of ZAP-70 negativity in *VH* unmutated cases with high-risk genomic aberrations. The current data suggest that 17p or 11q deletions, affecting critical cancer genes such as *TP53* and *ATM* (or possibly other genes in 11q*22-q23*), may lead to an inherent survival advantage independently of ZAP-70 mediated effects in CLL cells.

To evaluate the clinical implications of additional genetic high-risk features (*V3-21* usage, 11q or 17p deletion), we performed multivariate regression analyses. The *VH* mutation status, *V3-21* usage, the presence of high-risk genomic aberrations, but not ZAP-70 expression were identified as independent prognostic factors. Based on the multivariate regression analysis, we developed a model including the *VH* mutation status, *V3-21* usage, 17p deletion, and 11q deletion. The division in these prognostic categories allowed a good prediction of the clinical course in CLL. However, the association of ZAP-70 and the *VH* mutation status was strong in cases without additional genetic high-risk features, and the majority of *V3-21* cases showed high ZAP-70 expression irrespective of the *VH* mutation status. Based on these observations, we tested a second model including ZAP-70 expression and high-risk genomic aberrations. The prediction of the clinical course was similar to the model including *VH* mutation status, *V3-21* usage, and high-risk genomic aberrations. Therefore, a prognostic model based on ZAP-70 expression and genomic aberrations may be an alternative for the risk stratification of CLL patients, if *VH*-gene analysis is not available.

The current data reveal for the first time a characteristic pattern of important prognostic genetic features such as 11q deletion, 17p deletion, and *V3-21* usage in cases with discordant ZAP-70 and *VH* mutation status. ZAP-70 expression predicted an unfavorable clinical course in *V3-21* using cases irrespective of the *VH* mutations status, but did not predict the unfavorable clinical course of the majority of cases with unmutated *VH* accompanied by 11q or 17p deletion. According to the data presented in this study, we propose that high-risk genomic aberrations are important independent factors in addition to the *VH* mutation status and ZAP-70 expression for the risk stratification in CLL. If high-risk genomic aberrations are present, it appears to be important to consider this in addition to the prognostic impact of the *VH* status and ZAP-70 status for the prediction of the clinical course. In the absence of high-risk genomic aberrations the *VH* status and ZAP-70 status may have similar prognostic impact, and might therefore be alternatively applied. This is reflected by the fact, that high-risk genomic aberrations (11q and 17p) were selected as significant independent factors in both prognostic models (based on the ZAP-70 or *VH* status) constructed by multivariate analysis.

These observations have biologic implications with regard to the existence of different, BCR-dependent, and independent pathologic mechanisms in genetic subgroups of CLL, and prognostic importance with regard to the use of ZAP-70 as an outcome predictor. It is necessary to confirm these data in larger patient series. In addition, our data demonstrate the complexity of the assessment of the prognostic impact and interaction of multiple factors available in CLL, making further dissection of pathogenic pathways and the evaluation of combinations of prognostic factors in prospective trials mandatory.

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