Gene expression factors as predictors of genetic risk and survival in chronic lymphocytic leukemia

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ABSTRACT

Background

A variety of surrogate markers for genetic features and outcome have been described in chronic lymphocytic leukemia based on gene expression analyses. Previous studies mostly focused on individual markers and selected disease characteristics, which makes it difficult to estimate the relative value of the novel markers. Therefore, in the present study a comprehensive approach was chosen investigating 18 promising, partly novel expression markers in a well characterized cohort of patients with long clinical follow-up and full genetic information (*IGHV* status, genomic abnormalities).

Design and Methods

Expression markers were evaluated using real-time quantitative reverse transcriptase polymerase chain reaction in CD19$^+$-purified samples from 151 patients. Multivariate analyses were performed to test the markers' ability to identify patients at genetic risk and as prognostic markers in the context of established prognostic factors.

Results

For individual markers, *ZAP70* expression provided the highest rate (81%) of correct assignment of patients at genetic risk (*IGHV* unmutated, V3-21 usage, 11q- or 17p-), followed by *LPL* and *TCF7* (76% both). The assignment rate was improved to 88% by information from a four-gene combination (*ZAP70, TCF7, DMD, ATM*). In multivariate analysis of treatment-free survival, *IGHV* mutation status and expression of *ADAM29* were of independent prognostic value besides disease stage. With regards to overall survival, expression of *ATM*, *ADAM29*, *TCL1*, and *SEPT10* provided prognostic information in addition to that derived from clinical and genetic factors.

Conclusions

Gene expression markers are suitable for screening but not as surrogates for the information from genetic risk factors. While many individual markers may be associated with outcome, only a few are of independent prognostic significance. With regard to prognosis estimation, the genetic prognostic factors cannot be replaced by the expression markers.

Key words: chronic lymphocytic leukemia, markers, quantitative RT-PCR .

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The online version of this article has a supplementary appendix.

Introduction

The clinical course of chronic lymphocytic leukemia (CLL) is highly variable. Some patients require therapy immediately after diagnosis whereas others survive without treatment for several decades.[1-3] In order to develop risk-adapted strategies, prognostic factors are needed which allow the prediction of the individual clinical course. *IGHV* mutation status and genomic deletions at 11q22-q23 (11q-) and 17p13 (17p-) have been identified as strong and independent prognostic factors.[4-8] In addition, rearrangement of the *IGHV*-gene V3-21 has been associated with an unfavorable clinical outcome irrespective of the *IGHV* mutation status with survival times of the patients with this rearrangement being comparable to those of patients with unmutated *IGHV*.[9]

Based on these findings, gene expression parameters have been investigated for their association with genetic subgroups of CLL to reveal biological mechanisms and to identify potential surrogate markers for prognostic assessment. ZAP70, a ζ associated tyrosine kinase, has been broadly studied and was found to be a surrogate marker for unmutated *IGHV* status and for poor outcome.[10-13] However, there is discordance between ZAP70 expression and *IGHV* status in about 10 to 25% of cases of CLL. Discordance rates appear to be higher in specific genetic subgroups such as those using V3-21, or with 11q- or 17p-.[14,15] Additional potential surrogate markers for *IGHV* status have been suggested based on global gene expression studies.[16-18] Among these, lipoprotein lipase (*LPL*) showed promising results with regard to estimation of the *IGHV* mutation status and survival in purified[19] as well as in unpurified tumor samples.[20,21] Furthermore, a number of other individual markers showed an association with genetic subgroups, clinical course, or the pathogenesis of the disease.[22-29]

However, systematic comparative analysis is lacking since most of the studies focused on single markers or were based on small and heterogeneous cohorts of patients with incomplete genetic profiles. The aim of the present study was, therefore, to investigate the value of a broad range of novel and established surrogate markers, namely *ADAM29*, *ATM*, *CLLU1*, *DMD*, *GLO1*, *HCSL1*, *KIAA0977*, *LPL*, *MGC9913*, *PCDH9*, *PEG10*, *SEPT10*, *TCF7*, *TCL1*, *TP53*, *VIM*, *ZAP70*, and *ZNF2*, for their ability to predict the genetic risk of patients (defined by *IGHV* status, V3-21 usage, 11q-, and 17p-) and survival in multivariate analyses including established prognostic factors.

Design and Methods

Patients

A total of 222 CLL patients were included in the study at our institution. Peripheral blood samples were collected after informed consent. All cases matched the standard diagnostic criteria for CLL, no cases with t(11;14) were included. Real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR), *IGHV* mutation status analysis and fluorescence *in situ* hybridization (FISH) analysis were performed in all cases. In 151 cases, analyses were performed after CD19$^+$-cell purification using magnetic cell separation (MACS, Miltenyi, Bergisch Gladbach, Germany). In 105 cases, analyses were performed on unpurified peripheral blood mononuclear cell samples after Ficoll density separation. Thirty-four patients overlapped between both cohorts. The clinical characteristics and follow-up were available for 133 CD19$^+$ purified and 92 unpurified cases. Clinical and genetic characteristics are detailed in Table 1. All patients were untreated at the time of analysis and the median time from diagnosis to analysis was 1.2 months. Estimated median overall survival was 139 months after a median follow-up of 98 months in the overall cohort.

Analysis of genomic aberrations and IGHV status

FISH and *IGHV*-gene sequencing were performed as previously described.[4,7] A sequence homology cut-off of 98% was used to define the *IGHV* mutation status.

Real-time quantitative reverse transcriptase polymerase chain reaction

RNA was prepared and the RQ-PCR performed as reported elsewhere.[15] DNAse I digestion of total RNA was included to avoid contamination with genomic DNA. The TaqMan method (primers and probe) was used for quantification of all genes except for *ADAM29*, *MGC9913*, and *PCDH9*, for which the SYBR Green method was used. The primers for, and characteristics of, the candidate genes are listed in *Online Supplementary Table S1*. *GAPDH* was used as an endogenous control. Three peripheral blood samples from healthy donors were used, after CD19$^+$ purification, for standardization.

To test for gene expression levels in non-B cells of peripheral blood, the results for CD19-negative fractions from four CLL patients and three healthy donors were compared to those of the the respective positive fractions (*Online Supplementary Table S1*).

ZAP70 expression analysis by flow cytometry

For 72 cases included in the CD19$^+$ cohort, ZAP70 expression was measured by four-color flow cytometry (CD5, CD19, CD3/56, ZAP70) according to Crespo *et al.*,[11] as described previously.[14] Positivity was defined as a level greater than 20%.[11]

Statistical analysis

For the attribution of genetic risk, a high risk group (including all patients with an unmutated *IGHV* status or V3-21 usage or 11q- or 17p-) and a low risk group (*IGHV* mutated without usage of V3-21 and no 11q- or 17p-) were defined. Prediction of *IGHV* mutation status and genetic risk group stratification were performed with binary and multinomial logistic regression analysis including the expression levels of all genes. To assess the prediction error of the resulting predictor, ten repetitions of 10-fold cross-validation were used. Multivariate Cox proportional hazards models were used including the expression levels of all genes, Binet stage, age, and the genetic risk groups (*IGHV* status, V3-21 usage, 11q-, 17p-) for the analysis of overall survival and treatment-free survival times. Backward selection using Akaka's information criterion (AIC) was applied to exclude redundant or unnecessary variables. For purposes of comparison, a model based on the gene expression factors alone was calculated. To evaluate the prediction accuracy of the two models (the full model including all variables and the model including gene expression factors only), prediction errors over time were calculated using the loss function approach described elsewhere.[33,34] A measure of explained variation is derived by comparing the integrated prediction errors with the benchmark prediction error of survival prediction derived from Kaplan-Meier estimates. Kaplan-Meier estimates were used to compute marginal survival curves. Error estimation was done using ten repetitions of 10-fold cross-validation. Survival curves for censored data were estimated according to Kaplan and Meier. An effect was considered statistically significant when

(adjusted) *P* values were less than 5%. All statistical computations were performed with R, version 2.7.0, together with R packages multtest, version 1.20.0, pec, version 1.0.7, nnet, version 7.2-41, and Design, 2.1-1.[35]

Results

Fourteen out of 18 candidate genes were selected for investigation within a CD19+-purified cohort based on the results of a preliminary analysis of the 18 genes in an unpurified cohort (n=102) as well as on the expression pattern of the genes in CLL and non-CLL cells (*Online Supplementary Tables S1 and S2*). Four genes that were not overexpressed in non-CLL cells and did not have significant associations wtih survival or genetic subgroups were omitted. The CD19+ cohort comprised 151 cases and showed a representative distribution with respect to genetic prognostic factors (*Online Supplementary Figure S1*). In the following, only the results from the CD19+ cohort are reported.

Assignment of genetic risk

Assignment of *IGHV* mutation status was tested using logistic regression analysis. Best classifications among the candidate genes were obtained for *LPL*, *ZAP70*, and *TCF7* (*data not shown*). Correct assignment of the *IGHV* status was achieved in 83% of all cases when using *LPL* or *ZAP70* for classification, and in 75% when using *TCF7* (83%, 82%, and 73%, respectively, when assessed by ten repetitions of 10-fold cross-validation).

In addition to an unmutated *IGHV* status, V3-21 usage and deletions at 11q or 17p define poor risk subsets. Accordingly, genetic risk was defined by assigning all patients with an unmutated *IGHV* status, V3-21 usage, 11q- or 17p- to a high risk group, and patients with mutated *IGHV* without usage of V3-21, 11q- or 17p- to the low risk group (two-group risk model). According to this model, *ZAP70* provided the highest rate of correct classifications (81% of all cases), followed by *TCF7* and *LPL* (76% both) (Figure 1A-C) (81%, 76%, and 75%, respectively, after 10-fold cross-validation). Specifically, recognition of *IGHV* mutated V3-21-using cases as poor risk subset contributed to the superiority of *ZAP70* compared to *LPL* (Figure 1A-B): the majority of cases with V3-21 gene usage showed high levels of *ZAP70* expression, including four of six V3-21-using cases with mutated *IGHV*. These cases were classified false positive by *ZAP70* with regard to the *IGHV* mutation status but were correctly classified as being at high clinical risk (due to V3-21 usage). In contrast, *LPL* expression levels correctly predicted *IGHV* mutation status in seven of the nine cases using V3-21, i.e. mutated cases had predominantly low and unmutated cases high *LPL* expression and accordingly did not identify V3-21 usage as a marker of high clinical risk.

Marker combinations were tested to improve the classification according to the two-group risk model. Based on logistic regression analysis including the expression levels of all genes, a four-gene combination, based on *ZAP70*, *TCF7*, *DMD*, and *ATM* expression, was identified which provided correct classification in 88% of cases (Figure 1D) (85% after ten repetitions of 10-fold cross-validation).

The rates of discordance between *ZAP70* expression levels and *IGHV* mutation status have been reported to be higher for patients with 11q- and 17p-.[14] The relation between *IGHV* homology and gene expression within the 11q- and 17p- subgroups is detailed in Figure 1E-H for *ZAP70*, *LPL*, *TCF7*, and the four-gene combination according to the two-group risk model. The frequency of *IGHV* discordances within the 11q- and 17p- subsets was very low for *ZAP70*, *LPL*, and *TCF7* (3 of 24 patients with 11q-, 1 of 18 patients with 17p-), and even lower for the four-gene combination (2 of 24 patients with 11q-, 0 of 18 patients with 17p-).

Table 1. Patients' clinical and genetic characteristics divided into CD19+ purified and unpurified cases. Absolute numbers and % values are shown.

	CD19+ purified cases n=151	Unpurified cases n=105
Clinical feature		
male (%)	96 (63.6%)	62 (59%)
median age at diagnosis (range)	57 y (35-84)	58 y (34-88)
Binet stage (at diagnosis)	142	95
Binet stage A (%)	75 (52.8%)	48 (50.5%)
Binet stage B (%)	39 (27.5%)	30 (31.6%)
Binet stage C (%)	28 (19.7%)	17 (17.9%)
Survival information	133	92
events (death) (%)	47 (35.3%)	36 (39.1%)
median follow-up (months) (95% CI *)	97 (82 - 120)	96 (82 - 123)
median overall survival (months) (95% CI)	163 (124 - Inf)	115 (96 - Inf)
Treatment information	133	92
events (treatment) (%)	106 (80%)	65 (71%)
median treatment-free survival (months) (95% CI)	31 (24 - 47)	35 (22-54)
IGHV status		
IGHV unmutated (%)	92 (60.9%)	64 (61%)
V3-21 usage (%)	9 (6%)	12 (11.4%)
Genomic aberrations (FISH)		
13q as single abnormality (%)	52 (34%)	37 (35%)
trisomy 12 (%)	17 (11%)	18 (17%)
11q deletion (%)	24 (15.9%)	13 (12.4%)
17p deletion (%)	18 (11.9%)	13 (12.4%)
other abnormality (%)	8 (5%)	3 (3%)
no abnormality (%)	34 (23%)	21 (20%)

*CI: confidence interval.

Classification of the IGHV mutation status by ZAP70: polymerase chain reaction versus flow cytometry

For 72 cases of the purified cohort, ZAP70 protein expression was determined by flow cytometry, as described previously.[14] These results could be compared with transcript levels determined by RQ-PCR. Concordant results between flow cytometry and RQ-PCR were observed in 50 (69.4%) of the cases, while the results were discordant in 22 cases (30.6%). The discordant cases are listed in Table 2. Four of these cases showed high (i.e. false positive) mRNA levels by RQ-PCR in *IGHV* mutated cases including one case using the V3-21 gene. Another two were *IGHV* mutated cases with high (i.e. false positive) levels by flow cytometry. However, the majority of discordances (n=16) was related to *IGHV* unmutated cases with high ZAP70 levels by RQ-PCR but low levels (i.e. false negative) by flow cytometry.

Notably, half of these cases harbored one of the high risk

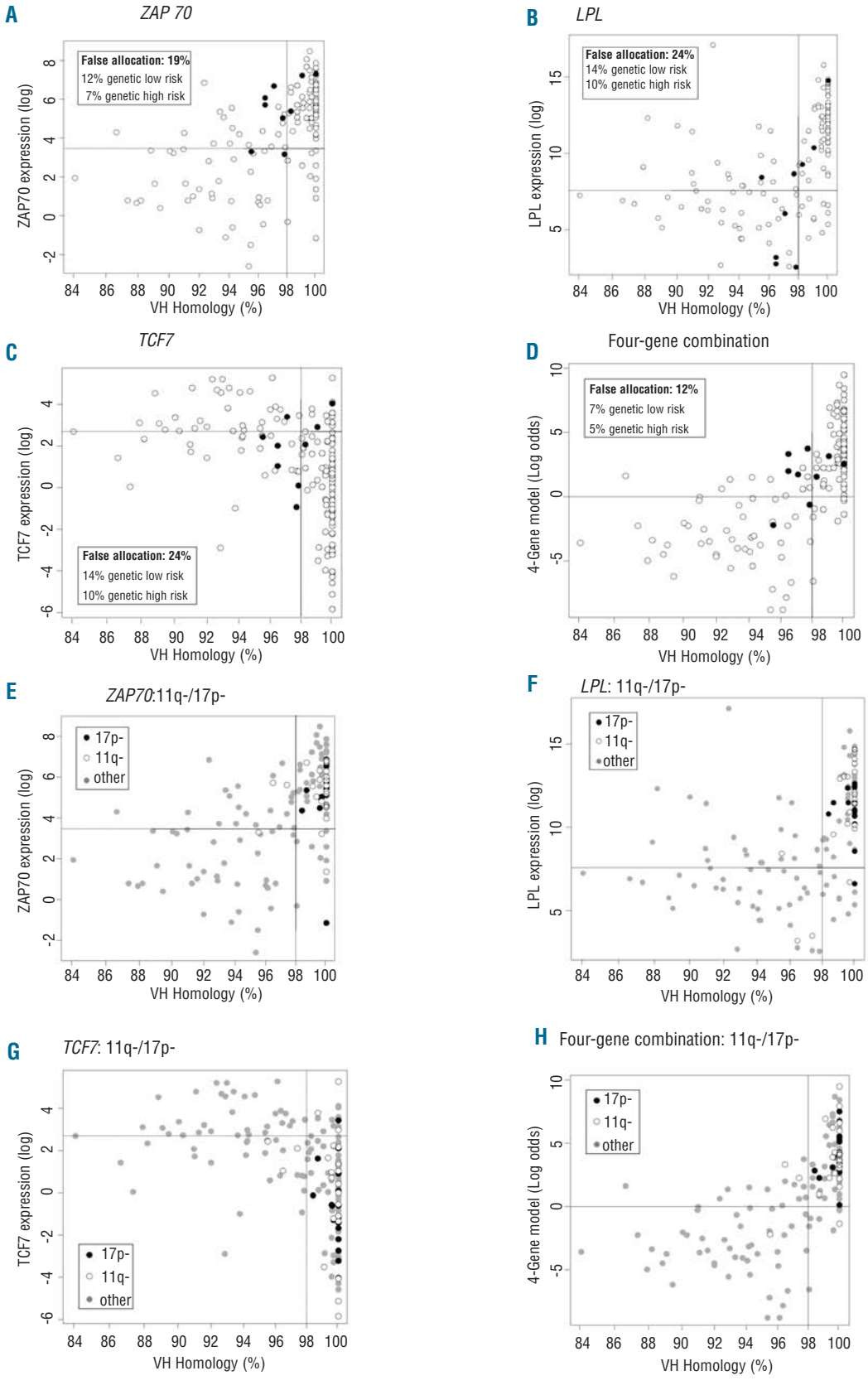

Figure 1. Patient distribution (n=151) according to marker expression and *IGHV* homology for *ZAP70* (A, E), *LPL* (B, F), *TCF7* (C, G), and the four-gene combination (D, H). Each circle represents one case. Y-axis: gene expression (logarithmic scale), X-axis: *IGHV* homology in %. Vertical line: 98% cut-off for the separation of the *IGHV* mutation subgroups. Horizontal line: gene expression cut-off for the separation of the high risk and low risk groups according to the logistic regression model prediction for the two-group risk model. A-D: all cases, V3-21 using cases indicated by filled circles. False allocations are given in % of the total cohort. E-H: all cases, 17p- and 11q- cases highlighted as shown.

aberrations 17p- or 11q- leading to a high proportion of *IGHV* misclassifications within these specific subsets (4 out of 12 cases for 11q-, 4 out of 9 for 17p-, compared to 10 out of 51 cases without 11q- or 17p-).

Prediction of treatment-free and overall survival

The prognostic value of the expression markers with regards to treatment-free survival and overall survival was studied using multivariate Cox regression analyses including the following variables: expression levels of all 14 candidate genes, clinical factors (age, stage), and the genetic factors (*IGHV* mutation status, V3-21 usage, 11q-, and 17p-) (full model; Table 3). The best estimation of treatment-free survival was achieved by a model including the variables *ADAM29*, *IGHV* mutation status, and Binet stage. Regarding the prediction of overall survival, a combined model consisting of clinical, genetic and gene expression markers was identified (Table 3). The most significant factors in this analysis were 17p-, Binet stage, and *ATM* expression. *IGHV* mutation status was not of significance in this model.

To explore the value of a prognostic model based only on gene expression factors, survival analyses were repeated excluding clinical and genetic factors (Table 4). According to this restricted model, treatment-free survival was best assessed by the expression levels of *DMD*, *TCF7*, and *ADAM29* with *DMD* being the strongest factor (*Online Supplementary Figure S2*). With regards to overall survival, *LPL* was identified as the strongest prognostic factor (*Online Supplementary Figure S3*); *ADAM29* and *ATM* were of additional prognostic impact. The prediction accuracy of the restricted model was compared to that of the full model (including genetic and clinical factors) based on their prediction errors in relation to a reference model without co-variables (i.e. Kaplan-Meier estimation) (Figure 2). With regards to treatment-free survival, the restricted and the full models showed similar prediction accuracy, indicating that the models were equivalent. With regards to overall survival, the gene expression model was inferior to the full model, with this latter showing a markedly lower rate of prediction errors.

Discussion

Numerous gene expression factors have been suggested as surrogate markers for genetic risk or prognosis in CLL patients.[10-29] In contrast to this progress in the identification of novel prognostic factors, systematic comparative analyses of these markers is lacking, and their significance in the context of established parameters and survival is not well elucidated. A comprehensive approach was chosen by van't Veer *et al.*,[21] who identified *LPL* as the best surrogate marker for *IGHV* mutation status with a prognostic value comparable to that of *IGHV* mutation status. However, their analyses were performed on non-purified tumor samples, limiting the results of this study since some of the markers (such as ZAP70) are strongly overexpressed in non-CLL cells. In the present study, the value of gene expression markers for the prediction of genetic subgroups and survival was assessed within a series of CD19$^+$-purified samples from patients using multivariate analyses in the context of established prognostic markers. The validity of the cohort for this type of analysis is suggested by the mature follow-up (median 97 months) and a repre-

Table 2. Characteristics of cases with discordance of ZAP70 expression as assessed by RQ-PCR and flow cytometry. Sorted by *IGHV* homology.

Case	ZAP70 RQ-PCR	ZAP 70 FACS	IGHV	IGHV gene	FISH	OS (mo.)	Status
1	20.8 (Pos.)	3.1 (Neg.)	86.57	DP58/hv3d1EG	Normal	113	Alive
2	24.0 (Pos.)	4.0 (Neg.)	94.14	V4-04	Normal	91	Alive
3	19.5 (Pos.)	17.0 (Neg.)	94.59	V3-07	12	89	Alive
4	108.0 (Pos.)	6.0 (Neg.)	97.00	V3-21	3q	23	Dead
5	4.1 (Neg.)	28.8 (Pos.)	84.02	V3-66	Normal	45	Alive
6	13.2 (Neg.)	78.2 (Pos.)	95.24	V4.30/4-31	12	52	Dead
7	43.7 (Pos.)	11.3 (Neg.)	98.20	V3-21	13q-	62	Alive
8	35.1 (Pos.)	0.6 (Neg.)	98.65	V1-03	13q-	289	Alive
9	380.0 (Pos.)	13.8 (Neg.)	99.55	V3-30	Normal	30	Alive
10	23,4 (Pos.)	13.9 (Neg.)	99.55	V3-48	17p-	26	Dead
11	29.8 (Pos.)	6.7 (Neg.)	99.66	V1-69	11q-, +8q, +3q		
12	34.0 (Pos.)	15.0 (Neg.)	99.66	V1-03	13q-	20	Alive
13	50.1 (Pos.)	8.8 (Neg.)	100.00	V3-09	11q-	28	Alive
14	25.7 (Pos.)	11.8 (Neg.)	100.00	V4-59	11q-	165	Dead
15	16.7 (Pos.)	9.0 (Neg.)	100.00	V2-05	11q-, 13q-	65	Dead
16	37.1 (Pos.)	15.9 (Neg.)	100.00	V1-18	17p-, 13q-		
17	53.1 (Pos.)	12 (Neg.)	100.00	V3-48	12	82	Alive
18	87.9 (Pos.)	17.2 (Neg.)	100.00	V3-09	Normal	20	Alive
19	89.6 (Pos.)	17.6 (Neg.)	100.00	V3-74	Normal	57	Alive
20	95.2 (Pos.)	13.1 (Neg.)	100.00	V1-69	13q-	53	Dead
21	99.7 (Pos.)	9.6 (Neg.)	100.00	DP58/hv3d1EG	17p-, 13q-	85	Dead
22	108.4 (Pos.)	18.5 (Neg.)	100.00	V1-08	17p-, 6q-	29	Alive

ZAP70 RQ: ZAP70 determined by RQ-PCR, cut-off for positivity: 16.68 according to the logistic regression model prediction for IGHV status. ZAP70 FACS: ZAP70 determined by flow cytometry, cut-off for positivity: 20%; OS: overall survival. Discordance between ZAP70 and the IGHV status is highlighted by blue letters.

sentative prognostic impact of established prognostic factors (*Online Supplementary Figure S1*). The best prediction of *IGHV* mutation status was equally achieved by *LPL* and *ZAP70*. However, a substantial percentage of patients (17%) were incorrectly assigned, indicating an incomplete overlap. When taking into account V3-21 usage, 11q-, and 17p- as additional poor risk subsets, *ZAP70* provided better classification rates compared to *LPL*, which was mostly due to the assignment of mutated V3-21 cases to poor clinical risk by *ZAP70*. This specific relation has been described earlier[14,15] and contrasts with LPL as detailed in this study. With regards to the prediction of patients at genetic risk, the assignment rate based on *TCF7* (also known as *TCF-1*) was similar to that achieved by *LPL*. In addition, *TCF7* was the strongest marker to be overexpressed in *IGHV*-mutated CLL, which is a novel finding and was reproduced at the protein level (*data not shown*). The association of *IGHV* with *TCF7* was superior to that with *ADAM29*, which is also overexpressed in *IGHV*-mutated CLL and has been described to improve the performance of *LPL* for *IGHV*-mutation status prediction.[19]

Table 3. Multivariate Cox regression analysis for treatment-free survival and overall survival including gene expression markers, clinical and genetic parameters (full model).

Variable	Effect	Hazard ratio for death (95% CI)	*P* value
Treatment-free survival			
ADAM29	Q75-Q25	0.55 (0.40, 0.74)	<0.001
IGHV status	unmutated *vs.* mutated	2.03 (1.33, 3.12)	0.001
Binet stage	B,C *vs.* A	1.95 (1.30, 2.92)	0.001
Overall survival			
17p-	yes *vs.* no	6.08 (2.17, 17.0)	<0.001
Binet stage	B,C *vs* A	3.56 (1.84, 6.90)	<0.001
ATM	Q75-Q25	0.42 (0.26, 0.68)	<0.001
ADAM29	Q75-Q25	0.34 (0.18, 0.66)	0.001
SEPT10	Q75-Q25	2.56 (1.46, 4.49)	0.001
V3-21	yes *vs* no	6.98 (2.06, 23.7)	0.002
TCL1	Q75-Q25	2.26 (1.28, 3.98)	0.005
Age	10-year change	1.52 (1.12, 2.08)	0.008

Effect: dimension of change of the variable as the basis for the calculation of the hazard ratio. Q75-Q25: change from the 25% to the 75% quartile of the gene expression distribution. CI: confidence interval.

Table 4. Multivariate Cox regression analysis for treatment-free survival and overall survival with gene expression markers as the only included variables (restricted model, excluding clinical and genetic factors).

Variable	Effect	Hazard ratio for death (95% CI)	*P* value
Treatment-free survival			
DMD	Q75-Q25	1.62 (1.21, 2.15)	0.001
TCF7	Q75-Q25	0.67 (0.50, 0.91)	0.01
ADAM29	Q75-Q25	0.67 (0.48, 0.93)	0.02
Overall survival			
LPL	Q75-Q25	2.97 (1.65, 5.34)	<0.001
ADAM29	Q75-Q25	0.56 (0.34, 0.93)	0.02
ATM	Q75-Q25	0.71 (0.52, 0.97)	0.03

Effect: dimension of change of the variable as the basis for the calculation of the hazard ratio. Q75-Q25: change from the 25% to the 75% quartile of the gene expression distribution. CI: confidence interval.

TCF7 is known to be as a T-cell specific transcription factor required for T-cell development, although its role in CLL is unknown. Animal models have suggested that it may have tumor suppressor-like function;[36] further functional studies in CLL would, therefore, be useful.

Classification of the *IGVH* mutation status among patients with 11q- or 17p- is of special interest, since increased misclassification of *IGHV* status was described within these subsets when using ZAP-70 determined by flow cytometry for classification.[14] However, in the present study, misclassification of *IGHV* status occurred infrequently within these subsets when using gene expression levels of *ZAP70*, as well as of *LPL* or *TCF7*, for classification (<10% of patients with 11q- or 17p-). Importantly, misclassification of patients with 11q- or 17p- occurred very rarely when using the four-marker combination (11q-: 2 of 24, 17p-: 0 out of 18) for *IGHV* assignment, underscoring the potential benefit of this classifier. However, although technically feasible, standardization of such a four-gene classifier would be challenging given the difficulties in standardization of the individual marker *ZAP-70*.[37-39]

Comparison of ZAP70 expression evaluated by FACS with gene expression levels revealed significant discordances between the results of the two methods (approximately 30%). The discordances were mainly *IGHV* unmutated cases being assigned false negative by FACS, pointing to a decreased sensitivity of the flow cytometric approach. This finding might reflect distinct biological properties of genetically high-risk CLL, such as post-translational down-regulation or enhanced protein degradation leading to reduced amounts of ZAP70 protein compared to mRNA. Alternatively, technical problems of flow cytometric detection might play a role such as usage of frozen samples or difficulties related to antibodies or procedures.[14,37-39] The RQ-PCR-based approach might, therefore, offer a sensitive and reproducible alternative.[40] However, the practicability of this approach is hampered by the need for cell purification prior to analysis.

Several of the investigated candidate genes have been proposed as novel prognostic factors in CLL.[10-13,19-21,23,26,28] Since most of the markers were identified based on their association with *IGHV* status, correlation with survival in univariate analysis is not unexpected. In multivariate approaches, *ZAP70* and *LPL* showed the potential to improve or substitute the information provided by *IGHV* status regarding treatment-free survival and overall survival.[11,21,39,41] These studies were, however, restricted to a few selected markers and did not account for the prognostic impact of genomic abnormalities and V3-21 usage which were, in contrast, included in the present study. In this study, the information from the candidate genes was not able to replace that from *IGHV* status and disease stage with regards to predicting treatment-free survival, but expression of *ADAM29* added independent prognostic information in the multivariate model. Therefore, *ADAM29* expression may be used for refined prediction of disease progression. Interestingly, a model based on the expression of only three genes provided a similar prognostic accuracy as that of the full model, thus offering a simplified tool for estimation of treatment-free survival.

The most validated established factors for prediction of overall survival are disease stage, *IGHV* mutation status, and

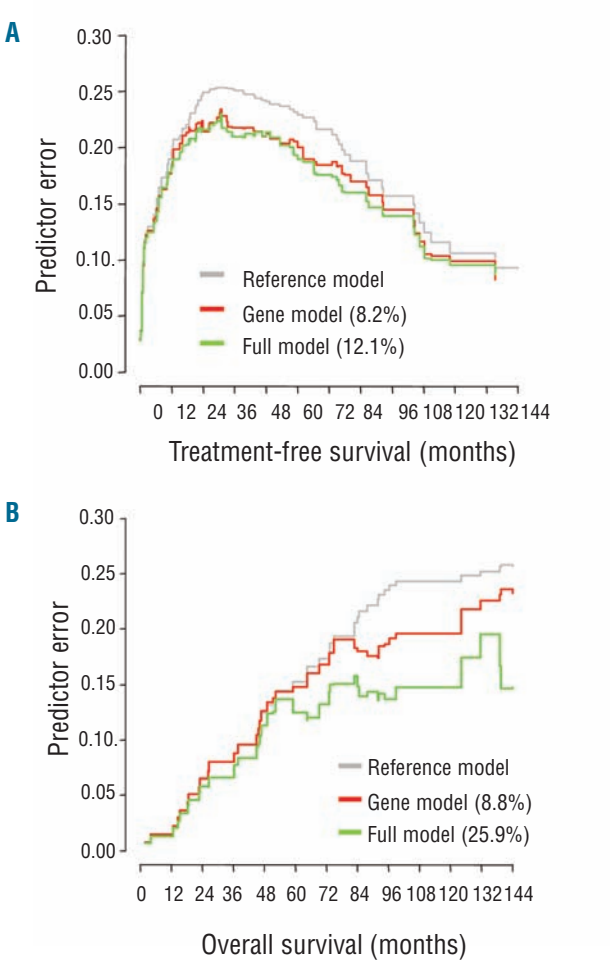

Figure 2. Prediction error curves for treatment-free survival (A) and overall survival (B) according to the restricted and full prognostic models. Restricted model (red): based on gene expression variables only; full model (green): including clinical and genetic factors; reference curve (gray): Kaplan-Meier estimation without additional variables. Curves below the reference curve indicate models with reduced prediction errors corresponding to higher explained variation values (% given in brackets for both models) and, therefore, higher prediction accuracy.

17p-. Multivariate analysis of overall survival including clinical and genetic factors resulted in a combined model predictor consisting of gene expression, clinical variables and genetic variables. This combined model was clearly superior to models based on gene expression factors alone or genetic factors alone (*data not shown*). Gene expression factors are, therefore, able to improve the estimation of overall survival provided by already established factors. The gene expression factors of additional impact were *ATM*, *ADAM29*, *TCL1*, and *SEPT10*. The quantitative relation between reduced levels of *ATM* expression and inferior overall survival is a novel finding and strongly suggests that this gene has a pathogenic role in CLL.

A close association between genomic loss at 11q22-q23 and reduced *ATM* transcript levels has been described[18,29] and can be interpreted as a gene dosage loss, supporting the postulated concept of *ATM* having a tumor suppressor function in CLL.[42-44] Selection of *ATM* expression instead of genomic deletion at 11q22-q23 in multivariate analysis indicates that quantitative transcript levels might reflect *ATM* dysfunction more precisely. *TCL1* over-expression in mice resulted in a disease resembling CLL, suggesting that *TCL1* might be directly involved in CLL transformation.[24] The prognostic impact of *TCL1* expression points to an ongoing pathogenic influence of *TCL1* deregulation during disease progression. *SEPT10* was significantly over-expressed in *IGHV*-unmutated CLL, confirming the findings of Bilban *et al.*[45] Extending those findings, Benedetti *et al.*[46] reported low *SEPT10* expression in V3-21 CLL, comparable to that in *IGHV*-mutated CLL patients. It, therefore, appears that *SEPT10* expression might be able to substitute for the survival information derived from *IGHV* mutation status but not for the information derived from V3-21 usage, as indicated by the multivariate overall survival model. *LPL* and *ZAP70* were not among the selected parameters for the overall survival model in line with previous findings that *ZAP70* lost prognostic significance in multivariate analysis when genetic factors were included.[14] Before the novel classifier can be recommended for further application, independent validation is required, which should best be performed within prospective clinical trials. The disadvantages of the classifier are its complexity, requiring analysis of a multitude of factors, and the need for cell purification prior to RQ-PCR analyses.

In conclusion, the novel gene expression markers are not a satisfactory surrogate for genetic risk factors but may be used for screening of genetic risk, which is best achieved by a marker combination. With regards to estimation of prognosis, the gene expression factors cannot replace established prognostic factors. However, a limited set of gene expression markers was of independent prognostic value and thus improved the prediction of treatment-free survival and overall survival. The potential value of the markers for future risk stratification strategies will depend on the clinical situation, as illustrated by the differential impact on treatment-free survival and overall survival and the influence of co-variates such as disease stage. The potential pathogenic implications of some genes such as *TCL1*, *ATM*, and *TCF7* warrant further functional investigation.

Authorship and Disclosures

DKi: designed and performed research, collected, analyzed and interpreted data, wrote the manuscript; AB: performed statistical analyses; CL, DW and CS: performed research; AB, TZ and AH: performed research, collected data; UJ, PL and RD-F: designed research; HD and SS: designed research, collected, analyzed and interpreted data, wrote the manuscript.

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