Dear author,

Please note that changes made in the online proofing system will be added to the article before publication but are not reflected in this PDF.

We also ask that this file not be used for submitting corrections.

Original Study

Prognostic and Predictive Effect of IGHV Mutational Status and Load in Chronic Lymphocytic Leukemia: Focus on FCR and BR Treatments

Andrea Visentin,[1,2] Monica Facco,[1,2] Carmela Gurrieri,[1] Elisa Pagnin,[1] Veronica Martini,[1,2] Silvia Imbergamo,[1] Federica Frezzato,[1,2] Valentina Trimarco,[1,2] Filippo Severin,[1,2] Flavia Raggi,[1,2] Edoardo Scomazzon,[1] Stefano Pravato,[1] Francesco Piazza,[1,2] Gianpietro Semenzato,[1,2] Livio Trentin[1,2]

Abstract

We performed a single-center retrospective study on 459 chronic lymphocytic leukemia patients diagnosed since 2000 to assess the prognostic and predictive role of immunoglobulin heavy chain (IGHV) mutational status and load. Unmutated IGHV patients had the shortest progression-free survival (PFS) and overall survival ($P < .0001$), whereas mutated IGHV patients experienced a long-term disease control after FCR (fludarabine with cyclophosphamide, and rituximab) or BR (bendamustine with rituximab) treatment, with PFS reaching a plateau, regardless of mutational load.

Background: Most important markers in chronic lymphocytic leukemia (CLL) are TP53 abnormalities, including mutations and deletions, and the mutational status of immunoglobulin heavy chain (IGHV) genes. However, some recent publications suggest that the IGHV mutational load could have a prognostic effect on CLL patients. **Patients and Methods:** We performed a single-center retrospective study on 459 patients with productive rearrangement of the B-cell receptor to evaluate the prognostic and predictive role of IGHV mutational status and burden within the germline sequence. In particular we focused on FCR (fludarabine with cyclophosphamide, and rituximab)- (64 naive and 30 relapsed) and BR (bendamustine with rituximab)-treated patients (17 naive and 61 relapsed). A cutoff value of 2% of difference within the IGHV germline was used to define the IGHV mutational status. **Results:** We reported that unmutated IGHV (U-IGHV) patients were characterized by a significant shorter progression-free survival (PFS) and overall survival ($P < .0001$) compared with mutated IGHV (M-IGHV) patients. Moreover, treatment-naive M-IGHV patients experienced a long-term disease control after FCR or BR, with PFS reaching a plateau regardless of mutational load. In our series the extent of IGHV gene mutation did not provide further relevant prognostic data over the mutational status. Relapsed patients showed dismal outcome with chemoimmunotherapy regardless of IGHV status or load. **Conclusion:** Our data, together with from those from the literature, confirmed the cutoff value of 2% to define the mutational status of IGHV gene and suggest that FCR/BR are good first-line treatment strategies for M-IGHV patients, whereas U-IGHV patients should be managed with B-cell receptor and/or BCL2 inhibitors.

Clinical Lymphoma, Myeloma & Leukemia, Vol. ∎, No. ∎, ∎-∎ © 2019 Published by Elsevier Inc.
Keywords: Prognostic factor, Predictive factor

A.V. and M.F. contributed equally to this work.

[1]Hematology and Clinical Immunology Unit, Department of Medicine, University of Padua, Padua, Italy
[2]Venetian Institute of Molecular Medicine, Centro di Eccellenza per la Ricerca Biomedica Avanzata, Padua, Italy

Introduction

Chronic lymphocytic leukemia (CLL), a clonal disorder characterized by the proliferation and accumulation of mature-appearing CD5-positive (CD5+) CD23+ B lymphocytes, is the most common hematological malignancy in Western countries. CLL is remarkably heterogeneous, with some patients never requiring treatment and others having highly aggressive and rapidly progressive diseases.[1]

The 3 most important CLL prognostic markers, that is, variables that identify subjects at higher risk of progression or death, are fluorescent in situ hybridization (FISH) analysis TP53 abnormalities, including mutation and deletion, and the mutational status of the variable region of the immunoglobulin heavy chain (IGHV) genes. Several efforts have been attempted to develop comprehensive approaches incorporating clinical, serum, genetic, and molecular markers with independent prognostic value into a single risk score for patients with CLL. In fact, these 3 markers have been combined with other clinical or biological variables in the most important and reliable prognostic models and indexes.[2-4] In addition, although cytogenetic and TP53 mutation can change over time because of a process of clonal evolution, IGHV mutational status is generally consider a stable marker,[5] even if some changes have rarely been described.[6] The cutoff value of a 2% deviation from, or <98% identity with, the corresponding germline sequence is generally adopted in the current clinical practice to discriminated between mutated IGHV (M-IGHV) and unmutated IGHV (U-IGHV) cases.

In recent years IGHV status and TP53 abnormalities have shown the capability to identify early relapse after first-line treatment with FCR (fludarabine with cyclophosphamide, and rituximab)[7-9] and BR (bendamustine with rituximab),[10,11] indicating their predictive role. Although TP53 mutations or 17p deletion identify most of relapsed/refractory (R/R) patients whose disease progressed early after chemoimmunotherapies,[10,12] the role of IGHV mutation is less clear and has been investigated less extensively in this setting. Recently, Jain et al showed that the absolute percentage deviation of IGHV mutation rather that a 2% cutoff predicts survival of CLL patients treated with FCR.[13] In particular, the 5-year progression-free survival (PFS) increase was 44%, 50%, and 86% in patients with 0.00%, 3.00%, and 7.00% of mutation within the germline sequence.[13]

In this single-center retrospective study, we analyzed 483 IGHV sequences derived from 459 patients with productive rearrangements of the B-cell receptor (BCR). We herein report the skewed **Q4 Q5** distribution of *IGHV*, *IGHD*, and *IGHJ* genes between M-IGHV and U-IGHV patients and we confirmed the threshold of 2% of *IGHV* gene sequence mutation within the germline sequence to define the mutational status of the *IGHV* gene, rather that the burden of the mutation. We also show that treatment-naive (TN) M-IGHV patients experienced a long-term disease control after FCR or BR, with PFS reaching a plateau, whereas this marker fails to show any predictive activity in the R/R setting.

Patients and Methods

Patients

Clinical data from 860 patients with CLL, according to iwCLL **Q6** 2008 criteria,[14] referred to the Hematology and Clinical Immu- **Q7** nology Unit of Padua University Hospital till 2017 were retrospectively reviewed for patients with productive rearrangement of the BCR.

Data included in the comparative analysis were sex, age, Rai and Binet stage,[14] need for chemotherapy, cytogenetic in FISH analysis,[15] IGHV mutational analysis,[16] and TP53 gene mutations[17,18] (details are provided in Supplemental Appendix A in the online version). Clinical data were recorded at CLL diagnosis whereas data on prognostic factors were collected according to international guidelines.[15]

Analysis of IGHV mutational status was performed within 6 months from diagnosis on peripheral blood CLL cells from fresh samples or from frozen purified CLL cells harvested in DMSO as **Q8** previously described.[19] Sequence homology <98%, from the corresponding germline gene, were considered mutated (M-IGHV), as opposed to unmutated (U-IGHV) cases.[19,20] The prognostic and predictive effect of IGHV mutations were assessed in a cohort of CLL patients diagnosed since 2000. High-risk FISH refers to 11q and/or 17p chromosome deletions, and TP53 abnormalities included 17p deletions and/or TP53 mutations.

This study was approved by the local research ethics committee and informed consent was obtained from all patients. The data are not publicly available because of restrictions from the ethics committee.

Treatment

According to NCI-WG 1996 and 2008 iwCLL guidelines, patients **Q9** were treated in the presence of progressive lymphadenopathy and/or hepato-splenomegaly, anemia, or thrombocytopenia due to bone marrow infiltration, systemic symptoms, lymphocyte doubling times <6 months, or refractory autoimmune cytopenia.[14] Fludarabine- or bendamustine-containing regimens, with or without rituximab were used as first-line treatment in fit patients, and chlorambucil with or without rituximab was used in elderly and/or unfit patients according to the treatment policy adopted at our center since 2000. FCR consisted of fludarabine 25 mg/m^2 with cyclophosphamide 250 mg/m^2 administered on days 1 to 3 of cycles 1 through 6 and rituximab 375 mg/m^2 on day 1 of cycle 1 and 500 mg/m^2 on day 1 of cycles 2 through 6.[21] BR consisted of bendamustine 90 mg/m^2 for TN and 70 mg/m^2 for R/R patients on days 1 and 2 of cycles 1 through 6 and rituximab 375 mg/m^2 on day 1 of cycle 1 and 500 mg/m^2 on day 1 of cycles 2 through 6.[22] Since January 2016 patients with TP53 abnormalities were treated with BCR inhibitors, ibrutinib, or idelalisib with rituximab.

Statistical Analysis

Categorical variables were compared using χ^2 test (for Rai stages and FISH analysis) or Fisher exact test (for age, sex, treatment, TP53, and IGHV), when appropriate. PFS and overall survival (OS) were calculated starting from the date of diagnosis to progression or death for any cause (event), respectively, or last known follow-up (censored).[14] PFS after first-line chemotherapy, including FCR and BR, was calculated as time from the beginning of first-line treatment to the date of progression or death (event) or last known follow-up (censored). Survival analyses were performed using the Kaplan−Meier method and the log rank test was used to compare survival curves between groups. A Cox regression model was used to estimate hazard ratios (HRs) and the proportional hazard assumption was tested for all Cox models. P values > .05

Variable	Population	Cohort	Treated	FCR Naive	BR Naive	FCR R/R	BR R/R
Table 1 — Clinical and Biological Characteristics of Patients							
Patients							
n	816	459	227	64	17	30	61
Sex							
Male	490 (60)	289 (63)	150 (66)	41 (64)	13 (76)	21 (70)	42 (69)
Female	328 (40)	170 (37)	77 (34)	23 (36)	4 (24)	9 (30)	19 (21)
Age at Diagnosis							
≥65	400 (49)	207 (45)	102 (45)	8 (13)	9 (53)	6 (20)	23 (38)
<65	416 (51)	252 (55)	125 (55)	56 (87)	8 (47)	24 (80)	38 (62)
Rai Stage							
0	367 (45)	211 (46)	70 (31)	22 (34)	7 (47)	8 (27)	15 (25)
I	220 (27)	133 (29)	32 (14)	24 (38)	4 (27)	6 (20)	21 (34)
II	122 (15)	69 (15)	45 (20)	12 (19)	3 (20)	13 (43)	17 (28)
III	19 (2)	9 (2)	9 (4)	2 (3)	1 (7)	1 (3)	2 (3)
IV	25 (3)	10 (2)	11 (5)	3 (5)	0 (0)	1 (3)	1 (2)
Treated							
Yes	326 (40)	227 (49)	141 (62)	30 (47)	6 (35)	23 (77)	37 (61)
No	490 (60)	232 (51)	86 (38)	34 (53)	11 (65)	7 (23)	24 (39)
FISH							
del 17p	38 (5)	28 (6)	25 (11)	9 (14)	3 (19)	4 (13)	9 (15)
del 11q	46 (6)	37 (8)	32 (14)	11 (17)	2 (13)	10 (33)	16 (26)
+12	58 (7)	41 (9)	25 (11)	10 (16)	3 (19)	2 (7)	6 (10)
Normal	145 (18)	92 (20)	30 (13)	10 (16)	4 (25)	4 (13)	9 (15)
del 13q	238 (30)	170 (37)	64 (28)	22 (34)	4 (25)	10 (33)	20 (33)
TP53 Abnormalties							
Yes	43 (5)	32 (7)	30 (13)	9 (14)	5 (29)	7 (23)	13 (21)
No	484 (61)	335 (73)	148 (65)	53 (83)	12 (71)	23 (77)	47 (79)
IGHV Homology							
>98	330 (40)	271 (59)	89 (39)	40 (62)	11 (65)	24 (80)	41 (67)
<98	225 (28)	188 (41)	138 (61)	24 (38)	6 (35)	6 (20)	20 (33)

Data are presented as n (%) except where otherwise noted.
Abbreviations: BR = bendamustine with rituximab; FCR = fludarabine with cyclophosphamide, and rituximab; FISH = fluorescence in situ hybridization; R/R = relapsed/refractory.

were considered as not significant. Statistical analysis was performed with R (an open source statistical package downloadable from http://www.r-project.org).

Results

Patient Features

Characteristics of the cohort are summarized in Table 1. We gathered data from 860 patients of whom 60% were male, 49% older than 65 years, 45% Rai stage 0 at diagnosis, and 40% required treatment during a median follow-up of 8.5 years. Four hundred fifty-nine subjects diagnosed since 2000 had 483 productive rearrangements of the BCR and 227 (49%) required CLL treatment: FCR was administered to 64 TN and 30 R/R cases, whereas BR was administered to 17 TN and 61 R/R patients.

As shown in Table 1 no significant differences were found between the whole population and the cohort with information on IGHV mutational status.

Prognostic Effect of IGHV Mutational Status

The prognostic activity IGHV mutational status was assessed in the whole cohort of 459 patients with 483 rearrangements diagnosed since 2000, we observed that not only *IGHV* genes, but also *IGHD* and *IGHJ* genes, showed a skewed distribution between M-IGHV and U-IGHV cases (see Supplemental Appendix A, Results, and Supplemental Figure 1 in the online version)

The median and the 10-year PFS were 2.88 versus 12.09 years and 14% versus 56% for U- and M-IGHV patients, respectively ($P < .0001$; Figure 1A). Instead, considering the mutational load of the *IGHV* gene the median PFS was 2.68, 3.25, and 10.45 years for patients with 0, 0.01% to 2%, and >6% mutations, but not reached for patients with 2.01%-4%, and 4.01%-6% mutations within the germline sequence ($P < .0001$; Figure 1C). As shown in Figure 1C the PFS curves of patients with 2%-4%, 4.01%-6%, and >6% mutations, that would have been classified as M-IGHV, have similar trends and were not statistically different ($P = .7455$), as

Figure 1 Prognostic and Predictive Implication of *IGHV* Mutational Status. (A and B) The Prognostic Role of *IGHV* Mutational Status on Progression-Free Survival (PFS) and Overall Survival (OS) of 459 Patients. (C and D) PFS and OS According to *IGHV* Mutational Load. In Particular, We Show That Mutated *IGHV* Patients Experience Longer Disease Progression and Survival Regardless of the Burden of Mutation. Kaplan—Meier Analysis of (E and F) PFS and (G and H) OS of the Predictive Role of *IGHV* Mutational Status and Load on 227 Treated Patients. We Focused on Treatment-Naive (TN) and Relapsed/Refractory (R/R) Patients Who Received FCR (Fludarabine with Cyclophosphamide, and Rituximab; I and M: 64 TN Patients; and L and N, 30 R/R Patients) and BR (O and Q: 17 TN Patients; P and R: 61 R/R Patients)
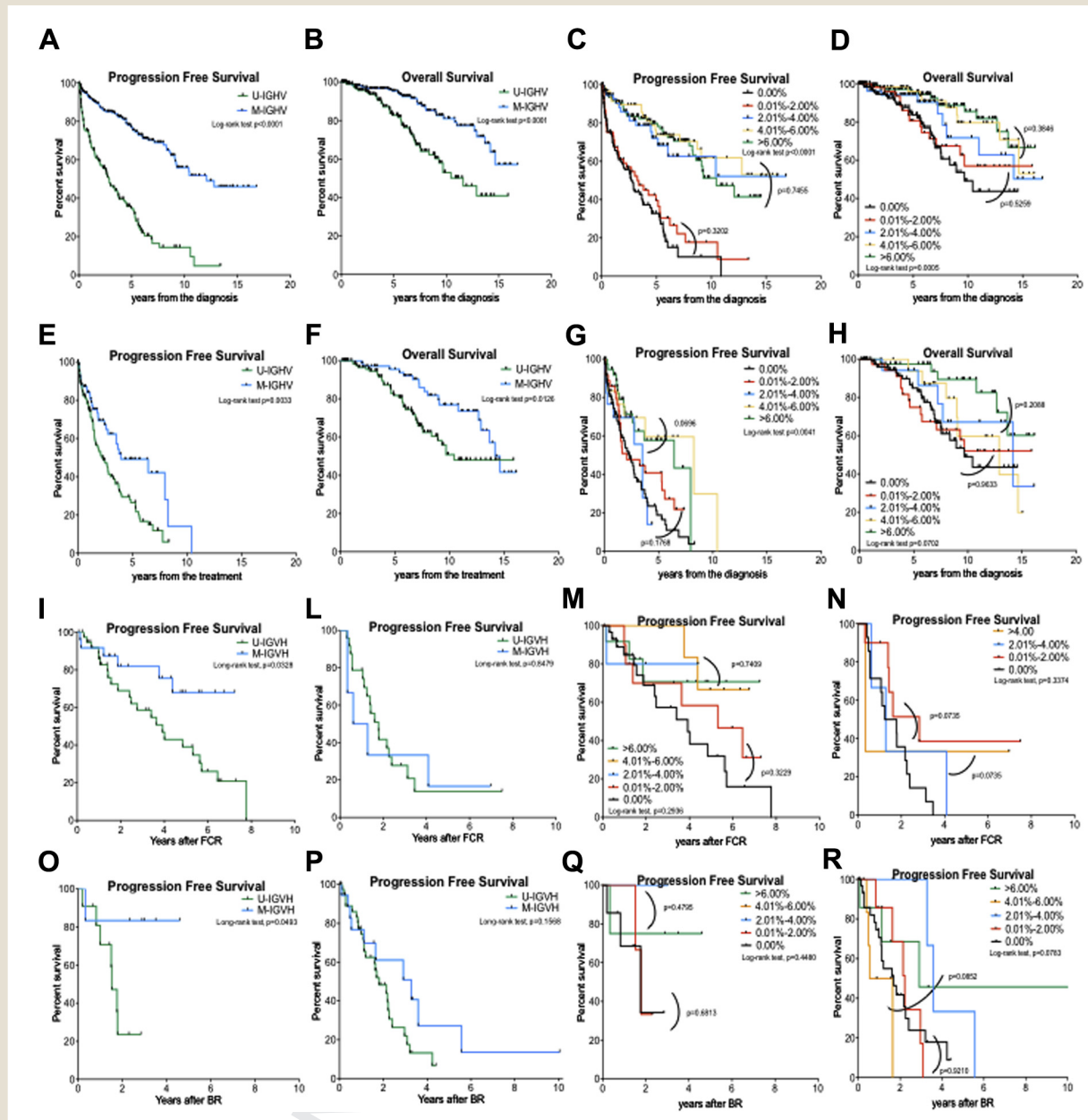
well as curves for patients with 0 and 0.01%-2% mutations (*P* = .3202; Figure 1C).

Unmutated IGHV subjects also had a shorter OS compared with M-IGHV. The median and the 10-year OS were 11.60 years versus not reached, and 53% versus 81%, respectively (*P* < .0001; Figure 1B). According to the mutational load the median OS was 9.69 for truly unmutated (0.00%) patients but not reached for

patients with 0.01% to 2%, 2.01% to 4%, 4.01% to 6%, and >6% mutations within the germline sequence (*P* = .0005; Figure 1D). However, the curves of patients with 2.01% to 4.00%, 4.01% to 6%, and >6% mutations, who would have been classified as M-IGHV, had superimposable trends (*P* = .3846; Figure 1D). Among U-IGHV patients, curves for patients with 0 and 0.01% to 2% mutations were not statistically different (*P* = .5259; Figure 1D).

In multivariate analyses, variables associated with a shorter OS were age older than 65 years, male sex, advanced stage, previous treatment, U-IGHV gene, 11q deletion, and TP53 abnormalities (Table 2).

Predictive Effect of IGHV Status

To evaluate the predictive strength of IGHV mutational status we analyzed 227 patients who required treatment during the follow-up since 2000. U-IGHV subjects had almost twofold increased risk of relapse and death after first-line therapy than M-IGHV (HRs, 1.78 and 1.97, respectively, $P < .0001$; Figure 1E, F). As shown in Figure 1G, H, the stratification of patients according to the extent of IGHV mutation had no effect PFS and OS, rather than the mutational status.

In particular, we focused on 64 TN and 30 R/R patients who received FCR (Figure 1I, J). The median PFS was 3.92 years for U-IGHV but not reached for M-IGHV patients ($P = .0328$) among the TN subgroup, whereas we did not find any difference in the R/R setting (1.78 and 0.95 years; $P = .8479$, respectively). Considering patients who were managed with BR (17 TN and 61 R/R; Figure 1M, N), IGHV mutational status provided predictive information only in previously untreated subjects but not in R/R cases ($P = .493$ and $P = .1568$, respectively). Moreover, as shown in Figure 1I, M, the PFS curves of M-IGHV subjects reached a plateau with both treatments. These data suggest a durable disease control and might be a cure with a short course, 6 cycles, of chemo-immunotherapy for M-IGHV patients.

We analyzed the effect of mutational load on the PFS and OS of all treated patients and those who received specifically FCR or BR. As shown in Figure 1K, L, Q, R, we did not find any statistical improvement of IGHV mutational load over the mutational status.

Discussion

In this single-center retrospective study we assessed the prognostic and the predictive role of IGHV mutational status compared with the IGHV mutational load in CLL patients followed in a single center. CLL cells express on their surface the BCR, made by surface immunoglobulins and CD79a and b, a crucial molecule for survival and functioning of normal B cells and many B-cell lymphoproliferative disorders. In 1999, 2 independent groups reported that CLL patients with higher (>2%) levels of somatic mutation in the

IGHV genes experienced longer PFS and OS.[20,23] This cutoff value was selected on the basis of the consideration that differences of IGHV genes up to 2% might have been related also to allele polymorphisms within the immunoglobulin loci. Three articles reported the capability of IGHV mutational status to predict the durability of response after FCR in TN subjects (Table 3).[7,8,10-12,21,22,31-33] The phase II trial, FCR300, of FCR as first-line treatment for patients with CLL showed a PFS of 53.9% for M-IGHV patients compared with 8.7% in U-IGHV patients after a median follow-up of 12.8 years.[7] PFS curves for M-IGHV subjects reached a plateau, suggesting sustained, long-term remission and, maybe, a cure. The subsequent phase III trial, CLL8, from the German CLL Study Group, showed that U-IGHV along with TP53 abnormalities had the strongest prognostic effect on PFS and OS.[21] Additionally, after almost 6 years of follow-up, more than 80% of M-IGHV patients were still alive, and the median OS for this subset was not reached.[9] Rossi and colleagues published an observational multicenter retrospective analysis of 404 CLL patients who received first-line FCR.[8] The combination of IGHV mutational status, 11q deletion, and 17p deletion allowed identification of a very low-risk category of patients, that accounted for 28% of all cases, featuring M-IGHV genes but neither 11q or 17p deletion. Most of these very low-risk patients (71%) remained free of progression after treatment and their hazard of relapse decreased after 4 years from FCR. The life expectancy of this subgroup (91% at 5 years) was superimposable to that observed in the matched normal general population.[8] Recently, Jain et al[13] showed that the absolute percentage deviation of IGHV mutation rather that a 2% cutoff predicts survival of CLL patients treated with FCR. In particular, the 5-year PFS increase was 44%, 50%, and 86% in patients with 0, 3%, and 7% of mutation within the germline sequence.[13] Instead in another multicenter international study the stratification of patients according to the mutational load did not reach an independent prognostic relevance, rather than the IGHV status.[24]

Data obtained from our 459 cases are in line with the previously mentioned reports (Figure 1I), confirming that U-IGHV patients have an aggressive disease whereas less than half of patients with M-IGHV required treatment and almost 80% were alive after 10 years of follow-up. Moreover, a homology >98% between the IGHV genes of the CLL clones and the normal counterpart represents a valid tool to discriminate groups with different prognostic

Table 2 Univariate and Multivariate Analyses of Overall Survival

	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	P	HR	95% CI	P
Male Sex	1.54	1.08-2.20	.0174	1.58	1.08-2.30	.0183
Age >65 Years	5.34	3.64-7.84	<.0001	5.11	3.40-7.69	<.0001
Rai Stage III-IV	3.36	1.57-7.22	.0019	2.31	1.34-3.98	.0025
Treated	2.21	1.56-3.15	<.0001	2.41	1.61-3.60	<.0001
11q-	2.57	1.30-3.70	.0056	2.30	1.19-4.10	.0079
TP53 Abnormality	3.03	1.42-6.45	.0040	2.25	1.28-3.98	.0051
U-IGHV	4.19	2.70-6.50	<.0001	3.70	2.42-5.68	<.0001

Abbreviations: HR = hazard ratio; 11q- = deletion 11q22-23; U-IGHV = unmutated immunoglobulin heavy chain.

Table 3 Summary of Most Relevant Studies With FCR and BR in TN and R/R Patients

Study	Phase	Patients	Follow-Up, Months	ORR, %	Median PFS, Months
FCR TN					
FCR300[7]	II				
M-IGHV/U-IGHV		88/126	154	n.a./n.a.	n.r./50
CLL8[21]	III				
M-IGHV/U-IGHV		197/111	71	93/91	n.r./44
CLL10[22]	III				
M-IGHV/U-IGHV		123/152	37	95/95	n.r./43
Rossi et al[8]	IV				
M-IGHV/U-IGHV		108/209	70	n.a./n.a.	n.r./48
Current study	IV				
M-IGHV/U-IGHV		40/24	53	n.a./n.a.	n.r./47
FCR R/R					
REACH[32]	III				
M-IGHV/U-IGHV		100/158	25	n.a./n.a.	n.a/n.a
Badoux et al[31]	II				
M-IGHV/U-IGHV		27/59	42	78/86	48/28
Current study	IV				
M-IGHV/U-IGHV		6/24	48	n.a./n.a.	22/11
BR TN					
GCCLSG[10]	II				
M-IGHV/U-IGHV		42/68	27	90/89	30/34
CLL10[22]	III				
M-IGHV/U-IGHV		87/183	37	97/95	55/34
GIMEMA[11]	IV				
M-IGHV/U-IGHV		66/57	24	89/82	n.a./n.a.
Current study	IV				
M-IGHV/U-IGHV		6/11	30	n.a./n.a.	n.r./18
BR R/R					
GCLLSG[12]	II				
M-IGHV/U-IGHV		25/51	24	78/59	18/14
ERIC[33]	IV				
M-IGHV/U-IGHV		40/106	37	80/77	31/21
Current study	IV				
M-IGHV/U-IGHV		20/41	30	n.a./n.a.	22/11

Abbreviations: BR = bendamustine with rituximab; CLL8 = ■ ■ ■; CLL10 = ■ ■ ■; ERIC = ■ ■ ■; FCR = fludarabine with cyclophosphamide, and rituximab; GCCLSG = ■ ■ ■; GCLLSG = ■ ■ ■; GIMEMA = ■ ■ ■; M-IGHV = mutated immunoglobulin heavy chain; n.a. = not available; n.r. = not reached; PFS = progression-free survival; REACH = ■ ■ ■; R/R = relapsed/refractory; TN = treatment-naive; U-IGHV = unmutated immunoglobulin heavy chain.

likelihood. In accordance with Morabito et al,[24] the extent of IGHV gene mutation did not reach any prognostic or predictive role over the IGHV status in our cohort of CLL patients (Figure 1I, M).

Treatment with FCR is associated with increased rates of hematological toxicity, infectious complications, and secondary malignancies, which are especially high in patients older than the age of 65 years.[21,25-27] In this regard, bendamustine, a chemotherapeutic agent that combines alkylating and purine antimetabolite properties, was effective and associated with a manageable toxicity profile when used for untreated patients with CLL.[28] The German CLL study group reported a phase II prospective study showing that BR is a safe and effective first-line therapy for CLL and, after a median

follow-up of 27 months, the median PFS was 33.9 months. In this work, the authors showed that 17p deletion, but not IGHV status, was associated with a lower remission rate and a shorter duration of response.[10] Gentile et al published an international multicenter retrospective study on BR in previously untreated CLL patients.[11] After median follow-up of 24 months the PFS was shorter for patients with a CIRS score >7, U-IGHV, 17p deletion, and BR dose intensity <80%.[11]

In our study, in accordance with data reported by Gentile et al,[11] we observed a longer PFS in M-IGHV patients treated with BR compared with U-IGHV patients (Figure 1M; $P = .0493$), rather that the burden of IGHV mutations. Interestingly, the PFS curves

of M-IGHV TN patients managed with either FCR or BR reached a plateau (Figure 1I, M), suggesting a durable disease control, a short course, 6 cycles, of chemoimmunotherapy.

Recently, the first in class BTK inhibitor ibrutinib, has been evaluated compared with FCR and BR in 2 milestone clinical trials in previously untreated CLL patients.[29,30] Both trials highlighted that 6 cycles of FCR or BR were as active and effective as long-term ibrutinib therapy in M-IGHV CLL, whereas significant improvements were observed for U-IGHV patients. Our data together with those derived from clinical trials suggest that M-IGHV patients significantly benefit from first-line FCR or BR with long-term disease control (Figure 1I, M).

The FCR and BR regimens have also been evaluated in R/R CLL patients without clear benefit on M-IGHV rather than U-IGHV patients (Table 3).[12,31-33] In our study we focused on 30 and 61 R/R patients treated with FCR or BR, respectively. Among FCR-treated patients, the median PFS for relapsed patients was shorter than for TN patients (1.79 vs. 4.94 years; $P = .0190$; see Supplemental Figure 2A in the online version); whereas it was similar for patients who received BR (1.78 vs. 2.14; $P = .9219$; see Supplemental Figure 2B in the online version). Interestingly, 14 patients who received FCR as first-line treatment were managed with BR at first relapse (ie, second-line therapy). As shown in Supplemental Figure 2C in the online version at, the median PFS and OS for the sequential use of these 2 regiments was 7.6 and 18.28 years, respectively. Opposite in TN patients, we did not find any predictive role of either the IGHV mutational status and the mutational load in relapsed subjects. Since new target therapies, mainly ibrutinib and venetoclax, showed a remarkable improvement over chemoimmunotherapies in R/R patients within clinical trials and real-life studies, they have become the standard of treatment.[1]

Conclusion

In our single-center retrospective study with a very long follow-up, we confirmed the threshold of 2% IGHV gene sequence homology within the germline sequence to define the mutational status of the *IGHV* gene, rather that the burden of the mutation, and further support the evidence that previously untreated M-IGHV patients without TP53 abnormalities should be effectively managed with few cycles of chemoimmunotherapy, FCR or BR depending on age, comorbidities, and renal function. On the contrary, TN CLL patients with U-IGHV and/or TP53 abnormalities reached limited disease control and early progression after chemo-immunotherapy and, for these reasons should receive BCR inhibitors or venetoclax.[1]

Clinical Practice Points

- In our single-center retrospectively study we confirmed the well established prognostic information of IGHV mutation status and further support the evidence that previously untreated M-IGHV patients, regardless of extent IGHV mutation, should be effectively managed with few cycles of chemoimmunotherapy, FCR or BR depending on age and comorbidities.
- On the contrary, CLL patients with U-IGHV and/or TP53 abnormalities reach limited disease control and early progression

after chemoimmunotherapy and for these reasons they should be candidates for BCR and/or BCL2 inhibitors.

Supplemental Data

Supplemental data and figures accompanying this article can be found in the online version at https://doi.org/10.1016/j.clml.2019.03.002.

Uncited Reference

Suppl ref. 5.

References

1. Scarfo L, Ferreri AJ, Ghia P. Chronic lymphocytic leukaemia. *Crit Rev Oncol Hematol* 2016; 104:169-82.
2. International CLL-IPI working group. An international prognostic index for patients with chronic lymphocytic leukaemia (CLL-IPI): a meta-analysis of individual patient data. *Lancet Oncol* 2016; 17:779-90.
3. Delgado J, Doubek M, Baumann T, et al. Chronic lymphocytic leukemia: a prognostic model comprising only two biomarkers (IGHV mutational status and FISH cytogenetics) separates patients with different outcome and simplifies the CLL-IPI. *Am J Hematol* 2017; 92:375-80.
4. Visentin A, Facco M, Frezzato F, et al. Integrated CLL scoring system, a new and simple index to predict time to treatment and overall survival in patients with chronic lymphocytic leukemia. *Clin Lymphoma Myeloma Leuk* 2015; 15:612-20. e1-5.
5. Crombie J, Davids MS. IGHV mutational status testing in chronic lymphocytic leukemia. *Am J Hematol* 2017; 92:1393-7.
6. Osman A, Gocke CD, Gladstone DE. Change in IgHV mutational status of CLL suggests origin from multiple clones. *Clin Lymphoma Myeloma Leuk* 2017; 17:97-9.
7. Thompson PA, Tam CS, O'Brien SM, et al. Fludarabine, cyclophosphamide, and rituximab treatment achieves long-term disease-free survival in IGHV-mutated chronic lymphocytic leukemia. *Blood* 2016; 127:303-9.
8. Rossi D, Terzi-di-Bergamo L, De Paoli L, et al. Molecular prediction of durable remission after first-line fludarabine-cyclophosphamide-rituximab in chronic lymphocytic leukemia. *Blood* 2015; 126:1921-4.
9. Fischer K, Bahlo J, Fink AM, et al. Long-term remissions after FCR chemoimmunotherapy in previously untreated patients with CLL: updated results of the CLL8 trial. *Blood* 2016; 127:208-15.
10. Fischer K, Cramer P, Busch R, et al. Bendamustine in combination with rituximab for previously untreated patients with chronic lymphocytic leukemia: a multicenter phase II trial of the German Chronic Lymphocytic Leukemia Study Group. *J Clin Oncol* 2012; 30:3209-16.
11. Gentile M, Zirlik K, Ciolli S, et al. Combination of bendamustine and rituximab as front-line therapy for patients with chronic lymphocytic leukaemia: multicenter, retrospective clinical practice experience with 279 cases outside of controlled clinical trials. *Eur J Cancer* 2016; 60:154-65.
12. Fischer K, Cramer P, Busch R, et al. Bendamustine combined with rituximab in patients with relapsed and/or refractory chronic lymphocytic leukemia: a

789 multicenter phase II trial of the German Chronic Lymphocytic Leukemia Study
790 Group. *J Clin Oncol* 2011; 29:3559-66.
13. Jain P, Nogueras Gonzalez GM, Kanagal-Shamanna R, et al. The absolute percent
791 deviation of IGHV mutation rather than a 98% cut-off predicts survival of chronic
792 lymphocytic leukaemia patients treated with fludarabine, cyclophosphamide and
rituximab. *Br J Haematol* 2018; 180:33-40.
793 14. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and
794 treatment of chronic lymphocytic leukemia: a report from the International
795 Workshop on Chronic Lymphocytic Leukemia updating the National Cancer
Institute-Working Group 1996 guidelines. *Blood* 2008; 111:5446-56.
796 15. Hallek M. Chronic lymphocytic leukemia: 2015 update on diagnosis, risk strati-
797 fication, and treatment. *Am J Hematol* 2015; 90:446-60.
16. Langerak AW, Davi F, Ghia P, et al. Immunoglobulin sequence analysis and
798 prognostication in CLL: guidelines from the ERIC review board for reliable
799 interpretation of problematic cases. *Leukemia* 2011; 25:979-84.
17. Pospisilova S, Gonzalez D, Malcikova J, et al. ERIC recommendations on TP53
800 mutation analysis in chronic lymphocytic leukemia. *Leukemia* 2012; 26:1458-61.
801 18. Raponi S, Del Giudice I, Marinelli M, et al. Genetic landscape of ultra-stable
chronic lymphocytic leukemia patients. *Ann Oncol* 2018; 29:966-72.
802 19. Terrin L, Trentin L, Degan M, et al. Telomerase expression in B-cell chronic lym-
803 phocytic leukemia predicts survival and delineates subgroups of patients with the
same igVH mutation status and different outcome. *Leukemia* 2007; 21:965-72.
804 20. Hamblin TJ, Davis Z, Gardiner A, et al. Unmutated Ig V(H) genes are associated with a
more aggressive form of chronic lymphocytic leukemia. *Blood* 1999; 94:1848-54.
805 21. Hallek M, Fischer K, Fingerle-Rowson G, et al. Addition of rituximab to fludar-
806 abine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a
randomised, open-label, phase 3 trial. *Lancet* 2010; 376:1164-74.
807 22. Eichhorst B, Fink AM, Bahlo J, et al. First-line chemoimmunotherapy with
808 bendamustine and rituximab versus fludarabine, cyclophosphamide, and rituximab
809 in patients with advanced chronic lymphocytic leukaemia (CLL10): an interna-
tional, open-label, randomised, phase 3, non-inferiority trial. *Lancet Oncol* 2016;
810 17:928-42.
23. Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression
811 as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 1999; 94:
812 1840-7.

24. Morabito F, Shanafelt TD, Gentile M, et al. Immunoglobulin heavy chain variable
846
region gene and prediction of time to first treatment in patients with chronic
847 lymphocytic leukemia: Mutational load or mutational status? Analysis of 1003
848 cases. *Am J Hematol* 2018; 93:E216-9.
25. Visentin A, Gurrieri C, Imbergamo S, et al. Epidemiology and risk factors of
849 invasive fungal infections in a large cohort of patients with chronic lymphocytic
850 leukemia. *Hematol Oncol* 2017; 35:925-8.
26. Visentin A, Imbergamo S, Gurrieri C, et al. Major infections, secondary cancers
851 and autoimmune diseases occur in different clinical subsets of chronic lymphocytic
852 leukaemia patients. *Eur J Cancer* 2017; 72:103-11.
27. Visentin A, Compagno N, Cinetto F, et al. Clinical profile associated with in-
853 fections in patients with chronic lymphocytic leukemia. Protective role of immu-
854 noglobulin replacement therapy. *Haematologica* 2015; 100:e515-8.
28. Knauf WU, Lissichkov T, Aldaoud A, et al. Phase III randomized study of
855 bendamustine compared with chlorambucil in previously untreated patients with
856 chronic lymphocytic leukemia. *J Clin Oncol* 2009; 27:4378-84.
29. Woyach JA, Ruppert AS, Heerema NA, et al. Ibrutinib regimens versus chemo-
857 immunotherapy in older patients with untreated CLL. *N Engl J Med* 2018; 379:
858 2517-28.
30. Shanafelt TD, Wang V, Kay NE, et al. A randomized phase III study of ibrutinib
859 (PCI-32765)-based therapy vs. standard fludarabine, cyclophosphamide, and rit-
860 uximab (FCR) chemoimmunotherapy in untreated younger patients with chronic
lymphocytic leukemia (CLL): a trial of the ECOG-ACRIN Cancer Research
861 Group (E1912). Q19
31. Badoux XC, Keating MJ, Wang X, et al. Fludarabine, cyclophosphamide, and
862 rituximab chemoimmunotherapy is highly effective treatment for relapsed patients
863 with CLL. *Blood* 2011; 117:3016-24.
32. Robak T, Dmoszynska A, Solal-Celigny P, et al. Rituximab plus fludarabine and
864 cyclophosphamide prolongs progression-free survival compared with fludarabine
865 and cyclophosphamide alone in previously treated chronic lymphocytic leukemia.
J Clin Oncol 2010; 28:1756-65.
866 33. Cuneo A, Follows G, Rigolin GM, et al. Efficacy of bendamustine and rituximab
867 as first salvage treatment in chronic lymphocytic leukemia and indirect comparison
868 with ibrutinib: a GIMEMA, ERIC and UK CLL FORUM study. *Haematologica*
2018; 103:1209-17.
869

Supplemental Data

Supplemental Appendix A

SUPPLEMENTAL METHODS

Fluorescent in Situ Hybridization

Fluorescent in situ hybridization was performed on standard cytogenetic preparations from peripheral blood. The slides were hybridized with the multicolor probe sets LSI p53/LSI ATM, LSI D13S319/LSI 13q34/CEP12 and RP11-177O8 (Vysis-Abbott, Des Plaines, IL) according to the manufacturer's protocol. Three hundred interphase nuclei were analyzed for each probe. Accordingly with the literature, the cutoff for positive values (mean of normal control ± 3 SD) was 4% for centromere 12 trisomy, and 10% for deletion of 11q22.3, 13q14.3, and 17p13.1.[1,2]

Immune Globulin Heavy Chain Variable Gene Mutation

To perform immune globulin heavy chain variable IGHV studies, RNA was extracted from 2×10^6 B cells using the RNeasy Total RNA kit (Qiagen) and reverse transcribed using the SuperScript Preamplification System for first-strand cDNA synthesis (Life Technologies, Inc). The B-chronic lymphocytic leukemia (CLL) cell VH gene family was assigned as previously described[2] using a sense VH family-specific framework region primer in conjunction with the appropriate antisense CH primer. VH gene sequences were determined by amplifying 5 μL of the original cDNA using the appropriate VH leader and CH primers. Polymerase chain reaction (PCR) products were sequenced directly after purification with Wizard PCR Preps (Promega, Madison, WI) using an automated genetic analyzer (3130 ABI Applied Biosystems, Foster City, CA). Sequences were analyzed using IMGT/VQUEST and BLAST software[3] to detect VDJ junction. Sequences homology ≤98, from the corresponding germline gene, were considered mutated,

opposite to unmutated cases.[2,4] Most common stereotyped rearrangements were determined with ARResT.[6]

TP53 Mutational Analysis

The mutation hot spots of the *TP53* (exons 2-11, including splicing sites) genes were analyzed using PCR amplification and DNA direct sequencing according to Rossi et al.[6]
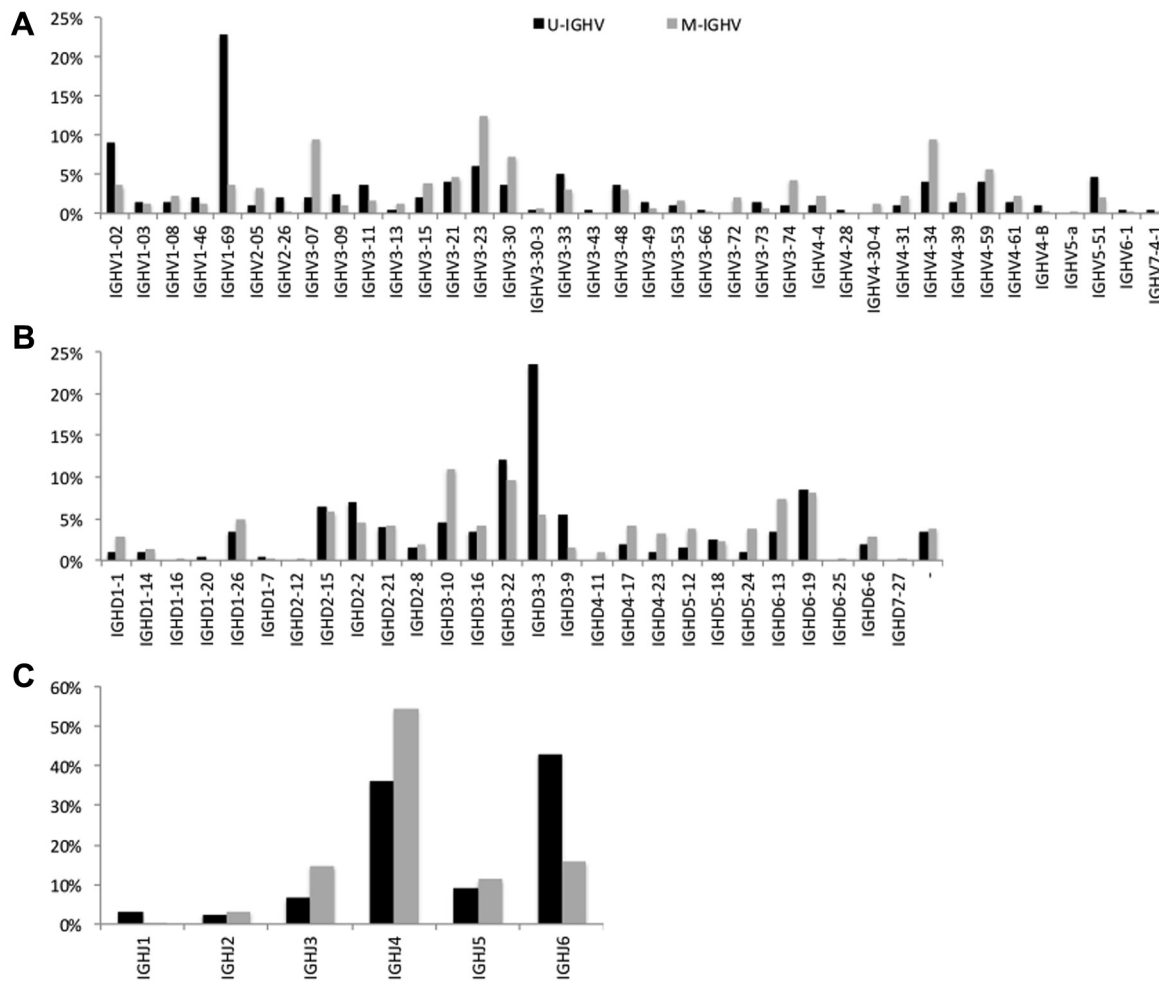
Supplementary Results

Immune Globulin Heavy Chain Variable, IGHD, and IGHJ Genes

Twenty-six patients had a stereotyped BCR and the most common subsets were number 2 (2%) and number 1 (1%). The median CDR3 length of our cohort was 13 amino acids (aa). However, we observed that patients belonging to unmutated CLL had longer CDR3 than mutated CLL patients ($P < .0001$). The average CDR3 lengths for unmutated IGHV (U-IGHV) and mutated IGHV (M-IGHV) patients were 22 and 15 aa, respectively. In our cohort, the most common IGHV genes were IGHV1-69 (11%), IGHV3-23 (10%), and IGHV4-34 (7%). As shown in Figure 1A, the distribution of these genes was skewed ($P < .0001$); in fact, the most rearranged genes in unmutated and mutated patients were IGHV1-69 (23% vs. 4%) and IGHV3-23 (6% vs. 13%), respectively. Considering IGHD genes, the most represented were IGHD3-3 (13%), IGHD3-22 (11%), and IGHD3-10 (8%). For these genes we also observed a nonrandom distribution between patients with U-IGHV and M-IGHV (Figure 1B; $P < .0001$). In fact, IGHD3-3 (24% vs. 6%) was the most common gene in the former group and IGHV3-10 in the latter (5% vs. 11%). Regarding IGHJ genes, the most common genes were IGHJ4 (47%), IGHJ6 (26%), and IGHJ3 (12%), and their distribution was unbalanced among patients (Figure 1C; $P < .0001$). In fact, IGHJ6 (43% vs. 16%) and IGHJ4 (36% vs. 55%) were the most common genes in U-IGHV and M-IGHV cases, respectively.

| | Supplemental Figure 1 | Additional Comparison of Survival Curves. (A) Comparison of Progression-Free Survival (PFS) After FCR (Fludarabine, Cyclophosphamide, and Rituximab) Treatment in Treatment-Naive (TN) versus Relapsed/Refractory (RR) Patients; the Median PFS for RR Patients Was Shorter Than for TN Patients (1.79 vs. 4.94 Years; *P* = .0190. (B) Comparison of PFS After BR (Bendamustine With Rituximab) Treatment in TN versus RR Patients; the Median PFS for RR Patients Was Similar to That of TN Patients (1.78 vs. 2.14 Years; *P* = .9219). (C) Survival Curves of 12 Patients Who Received FCR as First-Line Therapy and BR as Second-Line Therapy. The Median PFS and OS for the Sequential Use of These 2 Regimens Was 7.6 and 18.28 Years, Respectively |

| Supplemental Figure 2 | Prevalence and Distribution of *IGHV*, *IGHD*, and *IGHJ* Genes. (A) Histogram of *IGHV* Genes Between Mutated (*M-IGHV*) and Unmutated (*U-IGHV*) Patients. (B) and (C) Histograms of *IGHD* and *IGHJ* Genes; Comparison of *M-IGHV* and *U-IGHV* Patients, Respectively |
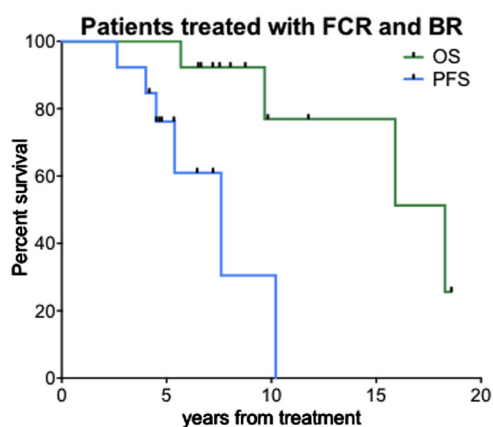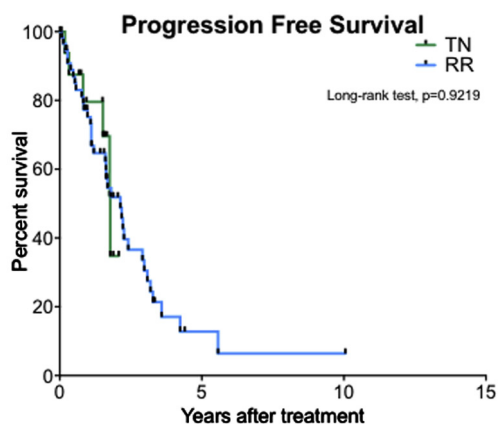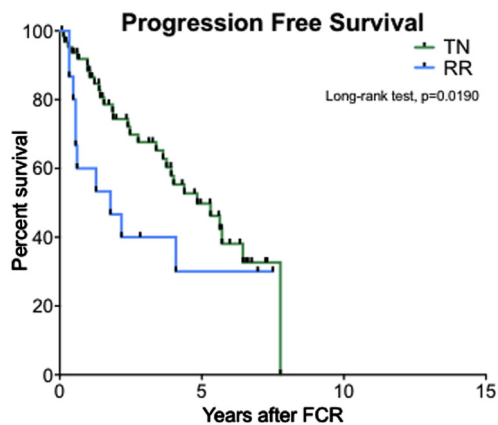
Supplemental References

1. Dohner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000; 343:1910-6.

2. Terrin L, Trentin L, Degan M, et al. Telomerase expression in B-cell chronic lymphocytic leukemia predicts survival and delineates subgroups of patients with the same IGVH mutation status and different outcome. *Leukemia* 2007; 21:965-72.

3. Brochet X, Lefranc MP, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic Acids Res* 2008; 36:W503-8.

4. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 1999; 94:1848-54.

5. Bystry V, Agathangelidis A, Bikos V, et al. ARResT/AssignSubsets: a novel application for robust subclassification of chronic lymphocytic leukemia based on B cell receptor IG stereotypy. *Bioinformatics* 2015; 31:3844-6.

6. Rossi D, Rasi S, Spina V, et al. Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. *Blood* 2013; 121:1403-12.