



Ferrata Storti Foundation

Biological and clinical implications of *BIRC3* mutations in chronic lymphocytic leukemia

Fary Diop,^{1*} Riccardo Moia,^{1*} Chiara Favini,¹ Elisa Spaccarotella,¹ Lorenzo De Paoli,¹ Alessio Bruscazzin,² Valeria Spina,² Lodovico Terzi-di-Bergamo,² Francesca Arruga,³ Chiara Tarantelli,⁴ Clara Deambrogi,¹ Silvia Rasi,¹ Ramesh Adhinaveni,¹ Andrea Patriarca,¹ Simone Favini,¹ Sruthi Sagiraju,¹ Clive Jabangwe,¹ Ahad A. Kodipad,¹ Denise Peroni,¹ Francesca R. Mauro,⁵ Ilaria Del Giudice,⁵ Francesco Forconi,^{6,7} Agostino Cortelezzi,⁸ Francesco Zaja,⁹ Riccardo Bomben,¹⁰ Francesca Maria Rossi,¹⁰ Carlo Visco,¹¹ Annalisa Chiarenza,¹² Gian Matteo Rigolin,¹³ Roberto Marasca,¹⁴ Marta Coscia,¹⁵ Omar Perbellini,¹⁶ Alessandra Tedeschi,¹⁷ Luca Laurenti,¹⁸ Marina Motta,¹⁹ David Donaldson,²⁰ Phil Weir,²⁰ Ken Mills,²¹ Patrick Thornton,²² Sarah Lawless,²⁰ Francesco Bertoni,⁴ Giovanni Del Poeta,²³ Antonio Cuneo,¹³ Antonia Follenzi,²⁴ Valter Gattei,¹⁰ Renzo Luciano Boldorini,²⁵ Mark Catherwood,²⁰ Silvia Deaglio,³ Robin Foà,⁵ Gianluca Gaidano^{1,°} and Davide Rossi^{2,°}

¹Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy; ²Institute of Oncology Research and Oncology Institute of Southern Switzerland, Bellinzona, Switzerland; ³Department of Medical Sciences, University of Turin & Italian Institute for Genomic Medicine, Turin, Italy; ⁴Università della Svizzera Italiana, Institute of Oncology Research, Bellinzona, Switzerland; ⁵Hematology, Department of Cellular Biotechnologies and Hematology, Sapienza University, Rome, Italy; ⁶Cancer Sciences Unit, Southampton Cancer Research UK and National Institute for Health Research Experimental Cancer Medicine Centre, University of Southampton, Southampton, UK; ⁷Division of Hematology, University of Siena, Siena, Italy; ⁸Department of Hematology Oncology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and University of Milan, Milan, Italy; ⁹Clinica Ematologica, DAME, University of Udine, Udine, Italy; ¹⁰Clinical and Experimental Onco-Hematology Unit, Centro di Riferimento Oncologico, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Aviano, Italy; ¹¹Department of Cell Therapy and Hematology, Ospedale San Bortolo, Vicenza, Italy; ¹²Division of Hematology, Azienda Ospedaliera Universitaria Policlinico-OVE, Catania, Italy; ¹³Hematology Section, Azienda Ospedaliera Universitaria Arcispedale S. Anna, University of Ferrara, Ferrara, Italy; ¹⁴Division of Hematology, Department of Oncology and Hematology, University of Modena and Reggio Emilia, Modena, Italy; ¹⁵Division of Hematology, Azienda Ospedaliera Universitaria Città della Salute e della Scienza and University of Turin, Turin, Italy; ¹⁶Section of Hematology, Department of Medicine, University of Verona, Verona, Italy; ¹⁷Department of Oncology/Haematology, Niguarda Cancer Center, Niguarda Ca Granda Hospital, Milan, Italy; ¹⁸Fondazione Policlinico Universitario A. Gemelli, Rome, Italy; ¹⁹Department of Hematology, Spedali Civili, Brescia, Italy; ²⁰Clinical Haematology, Belfast City Hospital, Belfast Health and Social Care Trust, Belfast, Northern Ireland, UK; ²¹Centre for Cancer Research and Cell Biology (CCRCB), Queen's University Belfast, Belfast, Northern Ireland, UK; ²²Department of Haematology, Beaumont Hospital, Dublin, Ireland; ²³Department of Hematology, Tor Vergata University, Rome, Italy; ²⁴Department of Health Sciences, University of Eastern Piedmont Amedeo Avogadro, Novara, Italy; ²⁵Department of Pathology, University of Eastern Piedmont Amedeo Avogadro, Novara, Italy.

**FD and RM contributed equally to this work

°GG and DR contributed equally to this work.

Haematologica 2020
Volume 105(2):448-456

Correspondence:

DAVIDE ROSSI
davide.rossi@eoc.ch

GIANLUCA GAIDANO
gianluca.gaidano@med.uniupo.it

Received: March 29, 2019.

Accepted: July 24, 2019.

Pre-published: August 1, 2019.

doi:10.3324/haematol.2019.219550

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/105/2/448

©2020 Ferrata Storti Foundation

Material published in *Haematologica* is covered by copyright. All rights are reserved to the Ferrata Storti Foundation. Use of published material is allowed under the following terms and conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>.

Copies of published material are allowed for personal or internal use. Sharing published material for non-commercial purposes is subject to the following conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>,

sect. 3. Reproducing and sharing published material for commercial purposes is not allowed without permission in writing from the publisher.



ABSTRACT

BIRC3 is a recurrently mutated gene in chronic lymphocytic leukemia (CLL) but the functional implications of *BIRC3* mutations are largely unexplored. Furthermore, little is known about the prognostic impact of *BIRC3* mutations in CLL cohorts homogeneously treated with first-line fludarabine, cyclophosphamide, and rituximab (FCR). By immunoblotting analysis, we showed that the non-canonical nuclear factor- κ B pathway is active in *BIRC3*-mutated cell lines and in primary CLL samples, as documented by the stabilization of MAP3K14 and by the nuclear localization of p52. In addition, *BIRC3*-mutated primary CLL cells are less sensitive to fludarabine. In order to confirm in patients that *BIRC3* mutations confer resistance to fludarabine-based chemoimmunotherapy, a retrospective multicenter cohort of 287 untreated patients receiving first-line FCR was analyzed

by targeted next-generation sequencing of 24 recurrently mutated genes in CLL. By univariate analysis adjusted for multiple comparisons *BIRC3* mutations identify a poor prognostic subgroup of patients in whom FCR treatment fails (median progression-free survival: 2.2 years, $P < 0.001$) similar to cases harboring *TP53* mutations (median progression-free survival: 2.6 years, $P < 0.0001$). *BIRC3* mutations maintained an independent association with an increased risk of progression with a hazard ratio of 2.8 (95% confidence interval 1.4-5.6, $P = 0.004$) in multivariate analysis adjusted for *TP53* mutation, 17p deletion and *IGHV* mutation status. If validated, *BIRC3* mutations may be used as a new molecular predictor to select high-risk patients for novel frontline therapeutic approaches.

Introduction

Nuclear factor- κ B (NF- κ B) signaling is a key component of the development and evolution of chronic lymphocytic leukemia (CLL).¹ Two NF- κ B pathways exist, namely the canonical and non-canonical pathways.² The former is triggered by B-cell receptor signaling via Bruton tyrosine kinase (BTK), while the latter is activated by members of the tumor necrosis factor (TNF) cytokine family.³ Upon receptor binding, the TRAF3/MAP3K14-TRAF2/BIRC3 negative regulatory complex of non-canonical NF- κ B signaling is disrupted, MAP3K14 (also known as NIK), the central activating kinase of the pathway, is released and activated to induce the phosphorylation and proteasomal processing of p100, thereby leading to the formation of p52-containing NF- κ B dimers. The p52 protein dimerizes with RelB to translocate into the nucleus, where it regulates gene transcription. BIRC3 (Baculoviral IAP Repeat Containing 3) is a negative regulator of non-canonical NF- κ B. Physiologically, BIRC3 (also known as cIAP2) catalyzes MAP3K14 protein ubiquitination in a manner that is dependent on the E3 ubiquitin ligase activity of its C-terminal RING domain. MAP3K14 ubiquitination results in its proteasomal degradation.⁴

B-cell neoplasia often pirates signaling pathways by molecular lesions to promote survival and proliferation. Although according to bioinformatics criteria *BIRC3* is one of the candidate driver genes of CLL, the functional implications of *BIRC3* mutations are partially unexplored.⁵⁻⁷ Furthermore, little is known about the prognostic impact of *BIRC3* mutations in CLL cohorts homogeneously treated first-line with fludarabine, cyclophosphamide, and rituximab (FCR).⁷

FCR is the most effective chemoimmunotherapy regimen for the management of CLL in young and fit patients devoid of *TP53* disruption.⁸ Survival after FCR is, however, variable, and is affected by the molecular characteristics of the CLL clone.⁹ Deletion of 17p and *TP53* mutations are present in most, but not all patients who are refractory to chemo-immunotherapy, which prompts the identification of additional biomarkers associated with early failure of FCR.¹⁰⁻¹²

Methods

Functional studies

The human CLL cell line MEC1, the splenic marginal zone lymphoma cell lines SSK41 and VL51, the mantle cell lymphoma cell lines MAVER-1, Z-138 and JEKO-1, the human HEK-293T cell line, as well as primary CLL cells were used in functional experiments. The entire non-canonical NF- κ B pathway was assessed by western blot analysis. Quantitative real-time polymerase chain reaction

(qRT-PCR) was utilized to analyze the non-canonical NF- κ B signature. Primary CLL were exposed to fludarabine and venetoclax for 24-48 h and apoptosis was measured using the eBioscience Annexin V Apoptosis Detection Kit APC (ThermoFisher). Details are supplied in the *Online Supplementary Methods*.

Cancer personalized profiling by deep sequencing

A retrospective multicenter cohort of 287 untreated CLL patients receiving first-line therapy with FCR was analyzed for mutations, including 173 patients from a previously published multicenter clinical series and 114 new patients not included in our previous report.¹⁰ The study was approved by the Ethical Committee of the Ospedale Maggiore della Carità di Novara associated with the Amedeo Avogadro University of Eastern Piedmont (study number CE 67/14). Further information is provided in the *Online Supplementary Methods*. A targeted resequencing gene panel was designed to include: (i) coding exons plus splice site of 24 genes known to be implicated in CLL pathogenesis and/or prognosis; (ii) 3'UTR of *NOTCH1*; and (iii) enhancer and promoter region of *PAX5* (size of the target region: 66627bp) (Table S1).^{6,7} The next-generation sequencing libraries for genomic DNA (gDNA) were constructed using the KAPA Library Preparation Kit (Kapa Biosystems) and those for RNA were constructed using the RNA Hyper Kit (Roche). Multiplexed libraries (n=10 per run) were sequenced using 300-bp paired-end runs on a MiSeq sequencer (Illumina) to obtain a coverage of at least 2000x in >90% of the target region (66627 bp) in 80% of cases (*Online Supplementary Table S2*). A robust and previously validated bioinformatics pipeline was used for variant calling (*Online Supplementary Appendix*).

Statistical analysis

Progression-free survival (PFS) was the primary endpoint. Survival analysis was performed with the Kaplan-Meier method and compared between strata using the log-rank test. To account for multiple testing, adjusted *P* values were calculated using the Bonferroni correction. The adjusted association between exposure variables and PFS was estimated by Cox regression. Internal validation of the multivariate analysis was performed using a bootstrap approach. Statistical significance was defined as a *P* value <0.05 (*Online Supplementary Appendix*).

Results

BIRC3 mutations associate with activation of non-canonical nuclear factor- κ B signaling

In order to map unique *BIRC3* mutations in CLL comprehensively, we compiled somatically confirmed variants identified in the current CLL study cohort with those identified in previous studies¹⁵ or listed in public CLL mutation catalogues (Figure 1A). Virtually all *BIRC3* mutations were frameshift mutations or stop codons clustering in two hotspot regions comprised between amino acids

367-438 and amino acids 537-564. *BIRC3* variants were predicted to generate aberrant truncated transcripts causing the elimination or truncation of the C-terminal RING domain of the *BIRC3* protein. The RING domain of

BIRC3 harbors the E3 ubiquitin ligase activity that is essential for proteasomal degradation of MAP3K14, the central activating kinase of non-canonical NF- κ B signaling. This observation points to non-canonical NF- κ B activation

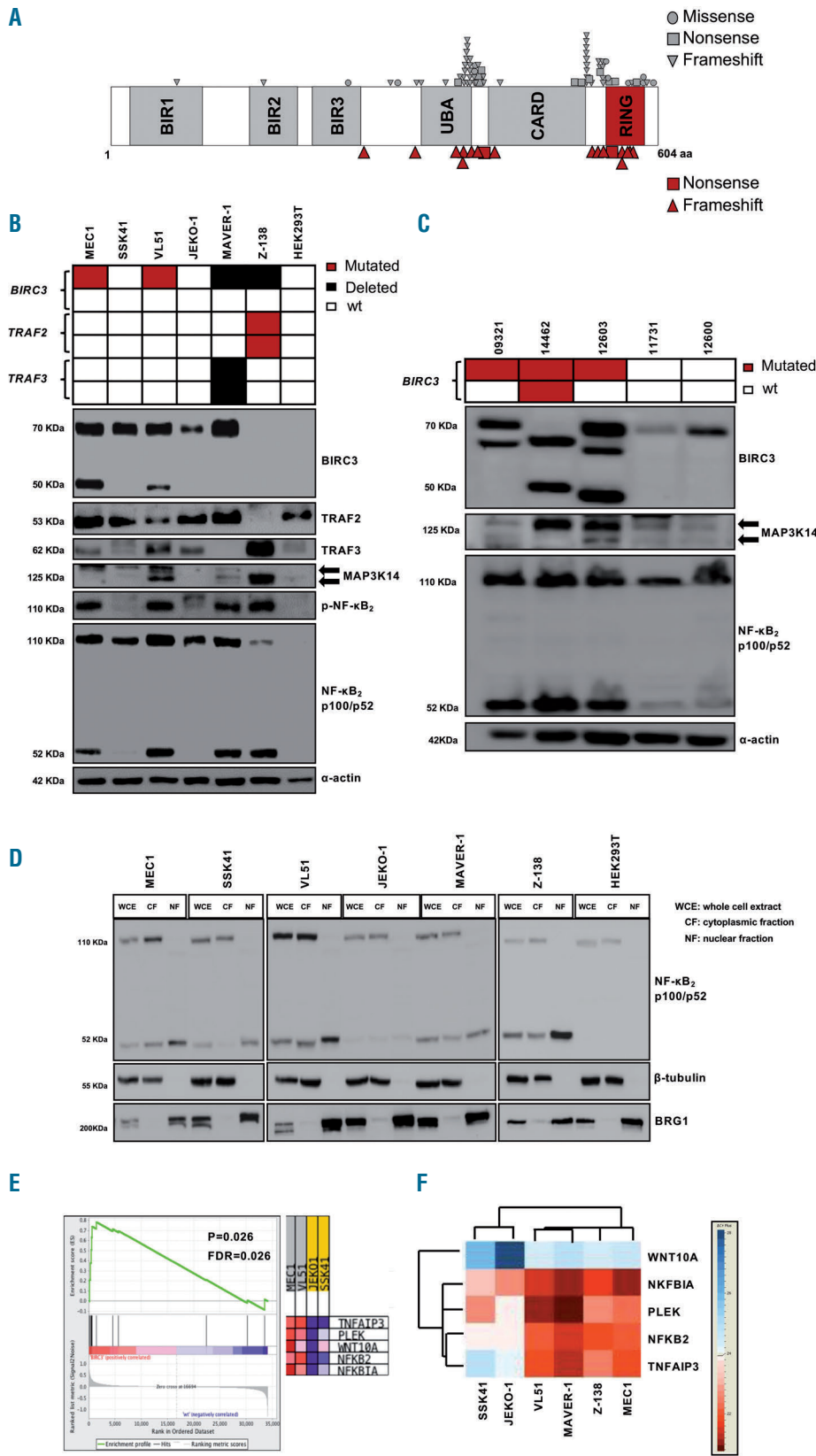


Figure 1. The non-canonical nuclear factor- κ B pathway is active in *BIRC3*-mutated chronic lymphocytic leukemia cell lines and primary samples. (A) Disposition of *BIRC3* mutations across the protein. The mutations identified by Landau et al.⁶, Puente et al.⁷ and from a public CLL mutation catalogue (COSMIC v85) are plotted in gray. Individual *BIRC3* mutations identified in the current studied cohort and in our previous study¹³ are plotted in red. (B) Western blot analysis of *BIRC3* protein expression and NF- κ B₂ activation and processing in the splenic marginal zone lymphoma (SMZL) cell lines SSK41, VL51 and in the chronic lymphocytic leukemia (CLL) cell line MEC1, carrying wildtype (wt) or disrupted *BIRC3*. The MAVER-1 and Z-138 cell lines were used as positive controls of non-canonical NF- κ B activation, harboring genetic activation of non-canonical NF- κ B signaling. The JEKO-1 and HEK 293T cell lines were used as negative controls for non-canonical NF- κ B signaling. α -actin was used as a loading control. Color codes indicate the gene status in each cell line. The aberrant *BIRC3* band expressed in MEC1 and VL51 cell lines corresponds in size to the predicted *BIRC3*-truncated protein, encoded by the mutant allele. (C) Western blot analysis showing *BIRC3* expression and NF- κ B₂ processing in purified primary tumor cells from five CLL and SMZL patients carrying wildtype or disrupted *BIRC3*. Color codes indicate the gene status in each cell line. The aberrant *BIRC3* bands in patients 09321, 14462 and 12603 correspond in size to the predicted *BIRC3*-truncated protein encoded by the mutant allele. α -actin was used as a loading control. (D) Western blot of whole cell extract, cytoplasmic or nuclear fractions of the SMZL and CLL cell lines probed for the NF- κ B₂ subunits p100 and p52. The MAVER-1 and Z-138 cell lines served as positive controls while the JEKO-1 and HEK 293T cell lines were used as negative controls. β -tubulin and BRG1 served as controls for the purity of the cytoplasmic and nuclear fractionations, respectively. (E) Gene set enrichment analysis score and distribution of non-canonical NF- κ B target genes along the rank of transcripts differentially expressed in the SMZL cell lines SSK41, VL51 and in the CLL cell line MEC1. The JEKO-1 cell line was used as a negative control. (F) Validation of expression of non-canonical NF- κ B target genes in the same SMZL and CLL cell lines as determined by quantitative real-time polymerase chain reaction analysis. Changes of gene expression were normalized to *GAPDH* expression; relative quantities were log₂ normalized to control samples (the mantle cell lymphoma cell line, JEKO-1).

through MAP3K14 stabilization as the predicted functional consequence of *BIRC3* mutations in CLL.

The non-canonical NF- κ B signaling was profiled by immunoblotting in B-cell tumor cell lines and primary CLL cells with different genetic make-up in the non-canonical NF- κ B pathway to verify whether *BIRC3* mutations lead to constitutive non-canonical NF- κ B activation. Additional genetic features of the above-mentioned cell lines and primary CLL cells are shown in *Online Supplementary Table S3*. In the VL51 splenic marginal zone lymphoma cell line and in the MEC1 CLL cell lines, both harboring endogenous truncating mutations of the *BIRC3* gene, non-canonical NF- κ B signaling was constitutively active, as documented by the stabilization of MAP3K14, phosphorylation of NF- κ B₂, its processing from p100 to p52, as well as the nuclear localization of p52 (Figure 1B-D). Consistent with the biochemical clues of non-canonical NF- κ B activation, the gene expression signature of the VL51 and MEC1 cell lines was significantly enriched in non-canonical NF- κ B target genes (Figure 1E, F). Non-canonical NF- κ B signaling in *BIRC3*-mutated cells was consistent with that in mantle cell lymphoma cell lines known to harbor a disrupted TRAF3/MAP3K14-TRAF2/BIRC3 negative regulatory complex by loss of TRAF3 or TRAF2.¹⁴ Like *BIRC3*-mutated cell lines, primary CLL samples harboring inactivating mutations of *BIRC3* also showed stabilization of MAP3K14 and NF- κ B₂ processing from p100 to p52 (Figure 1C), thus confirming that non-canonical NF- κ B activation is also a feature of primary cells harboring *BIRC3* variants. MAP3K14 stabilization is largely associated with *BIRC3* mutations. Indeed all seven cases harboring non-canonical NF- κ B genetic lesions showed either a strong or a slight MAP3K14 band, while, conversely, only one out of five cases lacking a non-canonical NF- κ B lesion had MAP3K14 expression (Fisher exact test, $P=0.01$).

MAP3K14 was genetically targeted by shRNA to test whether *BIRC3*-mutated cells are addicted to its stabilization. Compared to non-targeting shRNA, the most efficient anti-MAP3K14 shRNA-D resulted in a partial silencing of MAP3K14 and in decreased NF- κ B₂ processing from p100 to p52. This translated into a reduced cell viability of

the *BIRC3*-mutated VL51 cell line transduced with shRNA-D. This observation indicates that MAP3K14 stabilization is a vulnerability of *BIRC3*-mutated cells (Figure 2). In order to test the contribution of BTK to non-canonical NF- κ B signaling when it is activated through *BIRC3* mutations, *BIRC3*-mutated cell lines, as well as cell lines harboring a disrupted or competent TRAF3/MAP3K14-TRAF2/BIRC3 negative regulatory complex were treated with ibrutinib at different dosages and non-canonical NF- κ B signaling activation was probed by immunoblotting of the NF- κ B₂ processing from p100 to p52. Processing from p100 to p52 was unaffected by ibrutinib treatment in cell lines harboring *BIRC3* mutations (Figure 3) or a disrupted TRAF3/MAP3K14-TRAF2/BIRC3 negative regulatory complex, consistent with the notion that *BIRC3* mutations activate non-canonical NF- κ B by bypassing BTK blockade by ibrutinib.¹⁴

***BIRC3* mutations confer resistance to fludarabine in primary chronic lymphocytic leukemia cells**

We performed *in vitro* pharmacological studies on primary CLL cells to verify the vulnerabilities of *BIRC3*-mutated cells. CLL cells purified from patients carrying *BIRC3* mutations were treated with increasing doses of fludarabine. Drug-induced apoptosis was compared to that of samples harboring *TP53* mutations, which represent a control for fludarabine resistance. CLL cells devoid of genetic lesions in either *BIRC3* or *TP53* were used as a control for fludarabine sensitivity. The molecular characteristic of the *ex-vivo* CLL cells are listed in *Online Supplementary Table S4*.

BIRC3-mutated cells showed delayed fludarabine-induced cell death, as no response was observed after 24 h of treatment, at variance with *TP53*- and *BIRC3*-wildtype samples. At this time point, cell viability curves of *BIRC3*-mutated samples overlapped almost completely with those of *TP53*-disrupted samples, which are known to be fludarabine resistant (Figure 4A). At 48 h, the viability of *BIRC3*-mutated cells was lower than that of *TP53*-mutated samples, but higher than that of *TP53*- and *BIRC3*-wildtype samples (Figure 4B).

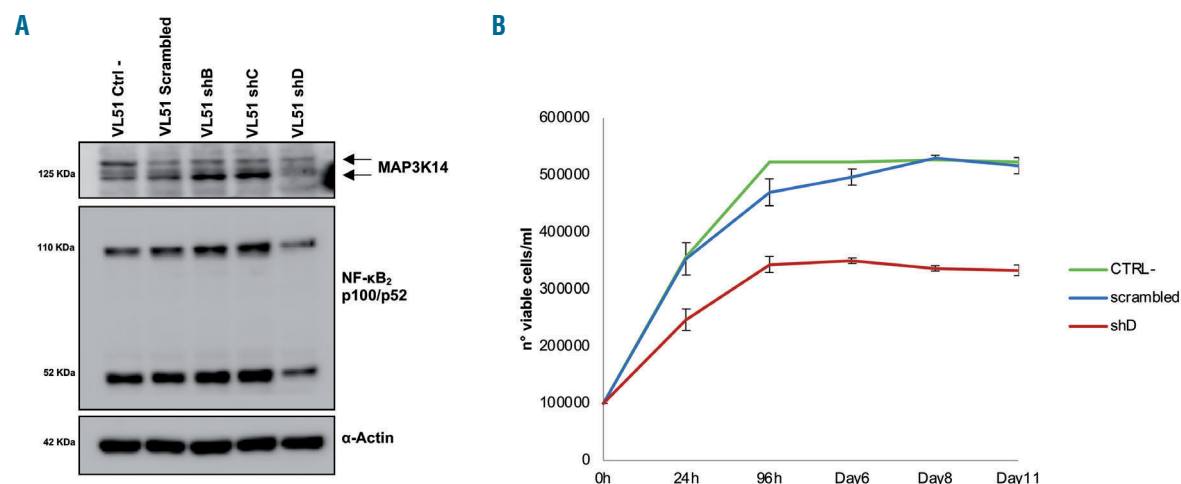


Figure 2. Knockdown of MAP3K14 by RNA interference in VL51 cells. (A) Western blot analysis for MAP3K14 expression and for NF- κ B₂ processing of p100 to p52. (B) VL51 cell viability assessed by trypan blue after transduction with lentiviral vectors expressing the shRNA-D_MAP3K14 (shD: in red), a scrambled shRNA (scrambled: in blue), and in non-transfected cells (CTRL: in green).

In order to assess whether *BIRC3* mutations interfere with apoptosis, primary CLL cells were treated with venetoclax. Venetoclax treatment resulted in a similar reduction of cell viability in *BIRC3*-mutated cells, *TP53*-mutated cells and *BIRC3/TP53*-wildtype cells (Figure 4C, D). Such divergent sensitivity to fludarabine and venetoclax of *BIRC3*-mutated CLL cells indirectly suggests that *BIRC3* mutations likely affect the upstream DNA damage response pathway rather than downstream apoptosis as a mechanism of inducing cell death.

Patients harboring *BIRC3* mutations are at risk of FCR failure

In order to confirm *in vivo* in patients that *BIRC3* mutations confer resistance to fludarabine-based chemoimmunotherapy, we correlated the *BIRC3* mutation status with PFS of CLL patients treated with FCR. Mutational profiling was performed in 287 patients who received first-line FCR. The baseline features of the study cohort were consistent with progressive, previously untreated CLL (Table 1). The median follow-up was 6.8 years, with

a median PFS and overall survival of 4.6 and 11.7 years, respectively (Table 1) consistent with observations in clinical trial cohorts.¹⁵ As expected, *SF3B1* and *NOTCH1* were the most frequently mutated genes identified in 13.9% and in 13.6% of patients, respectively, followed by *TP53* in 9.4% and *ATM* in 6.9% of patients. *BIRC3* was mutated in 3.1% of patients, reflecting the data reported in previous studies.^{6,7,13} Overall, 154/287 (53.6%) cases harbored at least one non-synonymous somatic mutation in one of the 24 CLL genes included in our panel (range, 1-5 mutation per patient), which is consistent with the typical mutational spectrum of CLL requiring first-line treatment (Figure 5, *Online Supplementary Table S5*).^{6,7,16}

By univariate analysis adjusted for multiple comparisons, among the genes analyzed in our panel, only *TP53* mutations (median PFS of 2.6 years; $P < 0.0001$) and *BIRC3* mutations (median PFS of 2.2 years; $P < 0.001$) (Figure 6A) were associated with significantly shorter PFS (Table 2). Following FCR treatment, the PFS of *BIRC3*-mutated patients was similar to that of cases har-

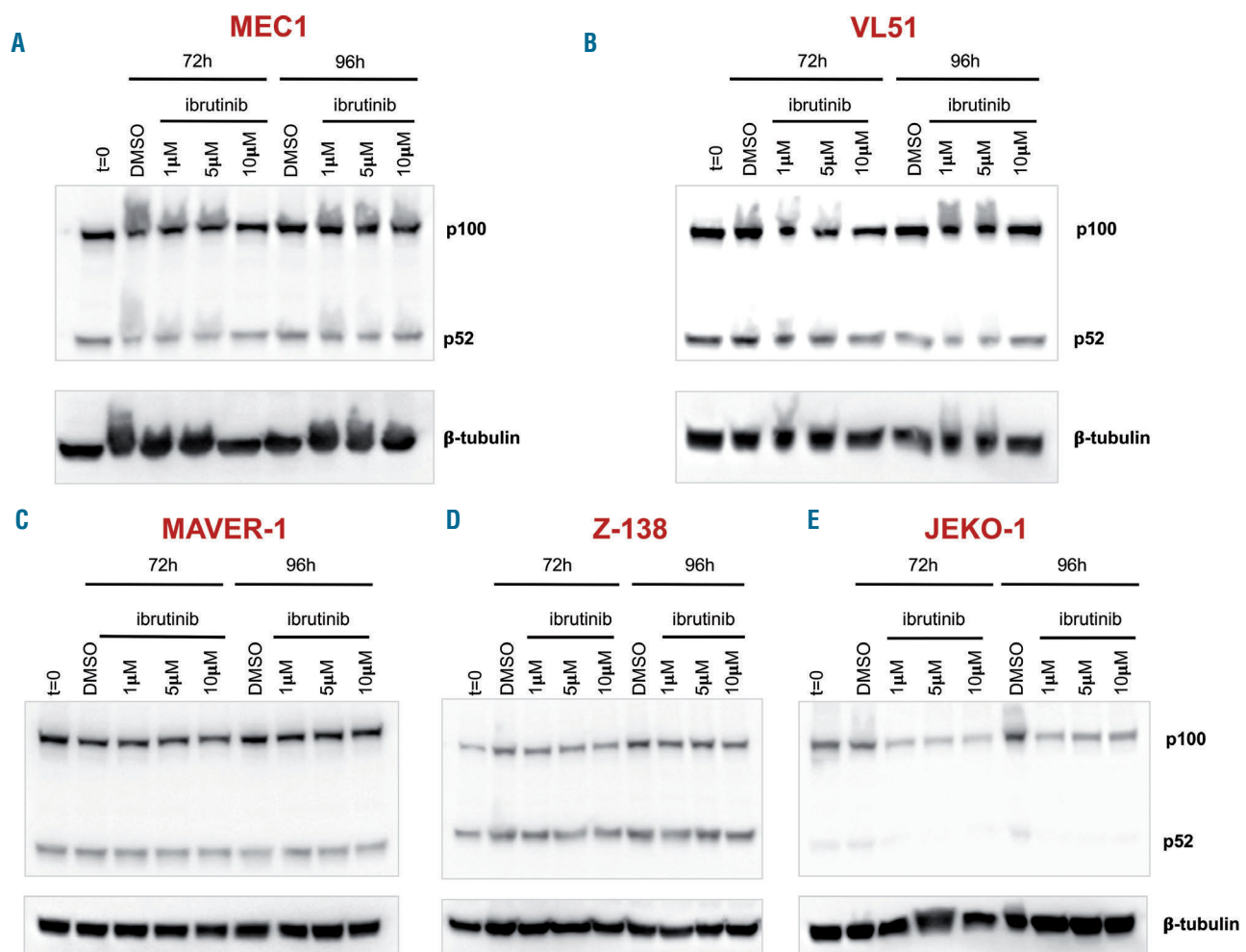


Figure 3. The non-canonical nuclear factor- κ B pathway is not switched off by ibrutinib in *BIRC3*-mutated cell lines. Western blot showing p100/p52 expression in (A) MEC1 and (B) VL51 cell lines that harbor *BIRC3* mutations. (C) MAVER-1 and (D) Z-138 cell lines, known to be affected by non-canonical NF- κ B pathway gene mutations and resistant to ibrutinib were used as positive controls. (E) The JEKO-1 cell line, known to be devoid of NF- κ B pathway gene mutations and sensitive to ibrutinib, was used as a negative control. All cell lines were treated with different concentrations of ibrutinib for 72 and 96 h. DMSO: dimethylsulfoxide.

boring *TP53* disruption (Figure 6B). Consistently, *BIRC3*-mutated patients had a lower likelihood of achieving complete response (22.2%) at the end of FCR compared to *BIRC3*-wildtype cases (76.7%; $P=0.001$). Well-known molecular prognostic biomarkers of CLL, such as unmutated *IGHV* gene status and 17p deletion also associated with a significantly shorter PFS, supporting the representativeness of the study cohort (Table 2). By multivariate analysis including variables showing a multiplicity-adjusted significant association with PFS, *BIRC3* mutations maintained an independent association with PFS, with a hazard ratio of 2.8 (95% confidence interval: 1.4-5.6; $P=0.004$) (Table 2).

Discussion

The results of this study provide evidence that: (i) *BIRC3* mutations are associated with activation of the non-canonical NF- κ B pathway and with resistance to fludarabine *in vitro*; and (ii) *BIRC3*-mutated patients, like cases harboring *TP53* disruption, are subject to failure of FCR chemoimmunotherapy.

The mere presence of somatic mutations is insufficient

to implicate a gene in cancer. Cancer geneticists and bioinformaticians differentiate “passenger” events, likely being randomly acquired, to distinguish them from mutations targeting candidate “cancer-driver” genes, likely implicated in the tumor biology, according to a statistical definition. Any given gene is labeled as a candidate “cancer driver” if it harbors somatic point mutations at a statistically significant rate or pattern in cancer samples. In CLL, more than 40 genes fulfill the statistical definition of a candidate “cancer driver”, including *BIRC3*, but few of them are biologically validated (i.e. *SF3B1*, *NOTCH1*, *TP53*, *ATM*, *FBXW7*).^{6,7,17-20} The *BIRC3* gene codes for a protein that ubiquitinates and negatively regulates the central activating kinase of the non-canonical NF- κ B pathway, namely MAP3K14 (also known as NIK).^{21,22} In lymphoid malignancies, the NF- κ B pathway is a pivotal and positive mediator of cell proliferation and survival.^{5,23,24} With regards to CLL, *BIRC3* mutations are absent in patients with monoclonal B-cell lymphocytosis, are rare at the time of diagnosis (3-4%), but are detectable in approximately 25% of fludarabine-refractory patients.¹³ In this study, we verified the biological consequences of *BIRC3* mutations, showing that they are associated with activation of the non-canonical NF- κ B pathway, that *BIRC3*-mutated lymphoid cells

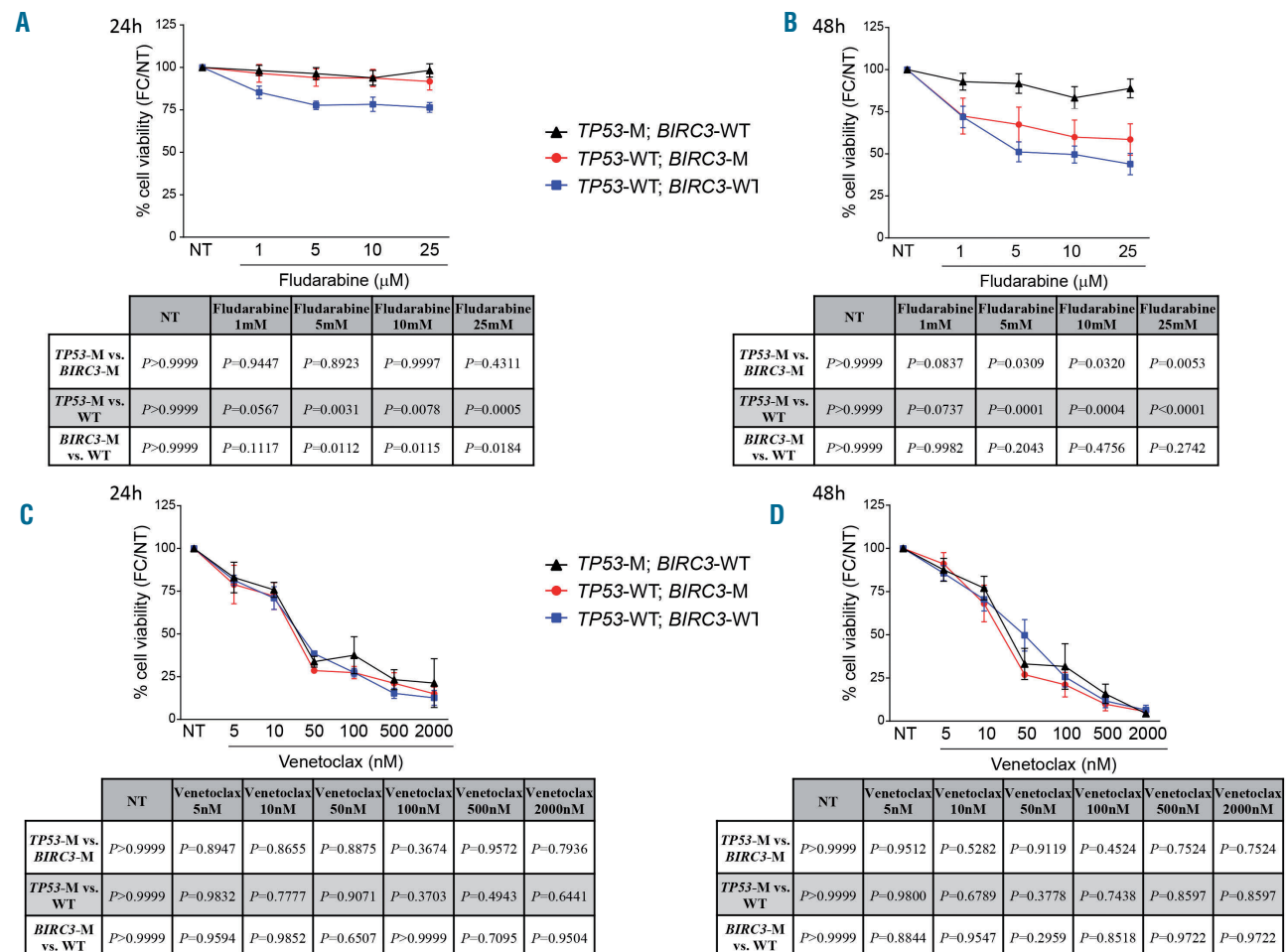


Figure 4. Responses of primary cells lines to fludarabine and venetoclax. (A-D) Viability of *BIRC3*-mutated (n=6 patients, red line), *TP53*-mutated (n=8 patients, black line) and wildtype (n=7 patients, blue line) primary CLL cells treated with different concentrations of fludarabine for 24 h (A) and 48 h (B) or venetoclax for 24 h (C) and 48 h (D). The pairwise *P* values are listed in the tables below the respective figures. M, mutated; WT, wildtype; NT, not treated.

are addicted to the non-canonical NF-κB pathway, and that *BIRC3*-mutated CLL are resistant to fludarabine both *in vitro* and in patients. It still remains to be clarified

whether NF-κB activation is the only molecular pathway that causes chemo-refractoriness in *BIRC3*-mutated CLL or whether other mechanisms are also involved.²⁴⁻²⁹

Table 1. Clinical data of FCR-treated chronic lymphocytic leukemia patients according to *BIRC3* mutational status.

Characteristics	Total	Number of patients (%)	<i>BIRC3</i> mutated patients (%)	<i>BIRC3</i> wildtype patients (%)
Male	N=287	198 (69.0%)	5 (55.6%)	193 (69.4%)
Female		89 (31.0%)	4 (44.4)	85 (30.6%)
Binet A	N=287	33 (11.5%)	1 (11.1%)	32 (11.5%)
Binet B-C		254 (88.5%)	8 (88.9%)	246 (88.5%)
<i>IGHV</i> mutated	N=280	100 (35.7%)	0 (0%)	100 (36.0%)
<i>IGHV</i> unmutated		180 (64.3%)	9 (100%)	171 (61.5%)
17p deletion	N=274	13 (4.7%)	0 (0%)	13 (4.7%)
No 17p deletion		261 (95.3%)	9 (100%)	252 (90.6%)
11q deletion	N=273	47 (17.2%)	5 (55.6%)	42 (15.1%)
No 11q deletion		226 (82.8%)	4 (44.4)	222 (79.9%)
13q deletion	N=273	111 (40.7%)	3 (33.3%)	108 (38.8%)
No 13q deletion		162 (59.3%)	6 (66.6%)	156 (56.1%)
Trisomy 12	N=272	50 (18.4%)	4 (44.4%)	46 (16.5%)
No trisomy 12		222 (81.6%)	5 (55.6%)	217 (78.1%)

Median follow-up (years)	6.8
Median PFS (years)	4.6
PFS % (7 years)	31.0%
Median OS (years)	11.7
OS % (7 years)	75.5%

PFS: progression-free survival; OS: overall survival; *IGHV*: immunoglobulin heavy variable gene.

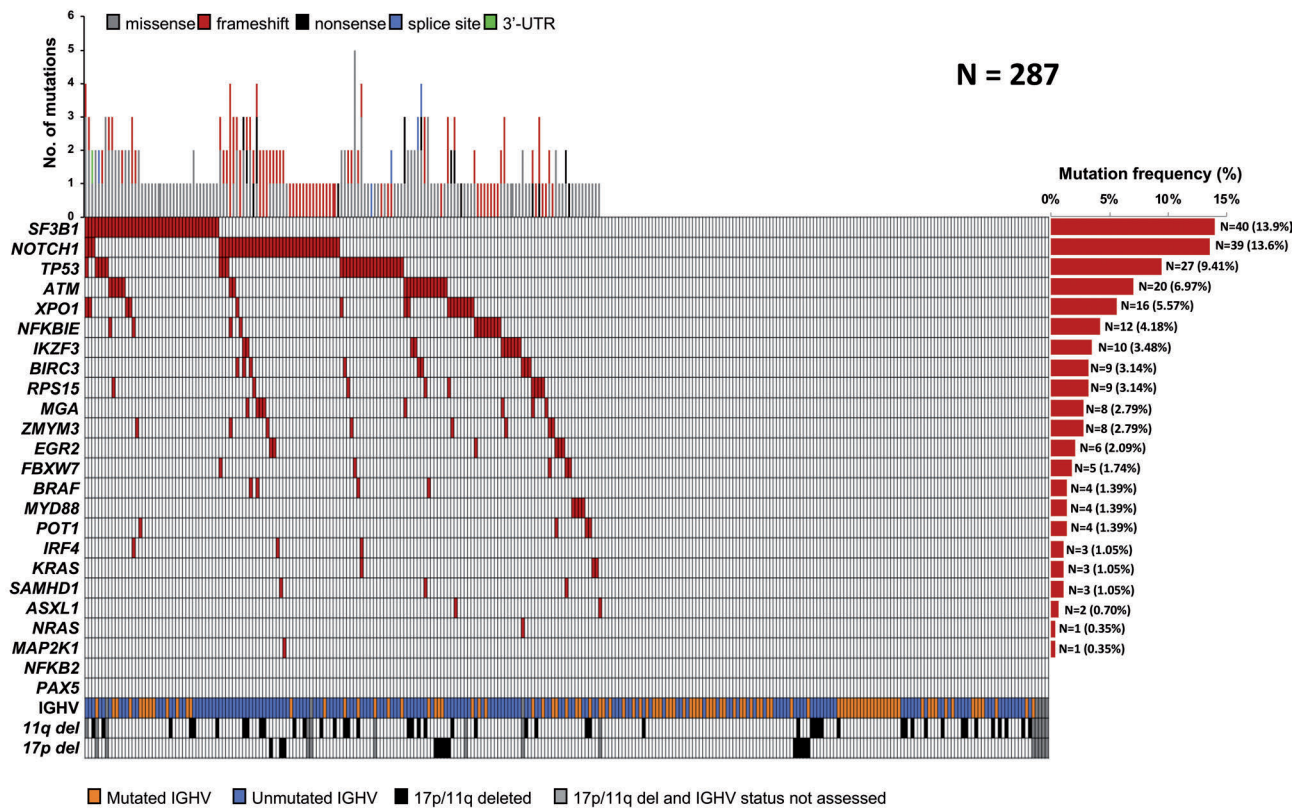


Figure 5. Mutational profile of the FCR-treated cohort. Case-level mutational profiles of 287 patients treated with FCR (fludarabine, cyclophosphamide, rituximab). Each column represents one tumor sample, each row represents one gene. The fraction of tumors with mutations in each gene is plotted on the right. The number and type of mutations in each patient are plotted above the heatmap. Mutations are highlighted in red. *IGHV* mutational status, 17p deletion and 11q deletion are plotted at the bottom of the heatmap.

The introduction of FCR was a breakthrough in the management of young, fit CLL patients, leading to improvements in both PFS and overall survival compared to those achieved with previous treatment regimens. In both clinical trials and real-life cohorts,¹⁰⁻¹² *IGHV* mutation status and *TP53* disruption emerged as strong predictors of poor response to FCR. However, these molecular biomarkers do

not fully capture all high-risk patients destined to relapse. We propose *BIRC3* mutations as a new biomarker for the identification of patients at high risk of FCR failure, similarly to cases harboring *TP53* disruption. If validated in independent series, *BIRC3* mutations may turn out as a new molecular predictor of FCR resistance that could be used to select patients to be treated with novel targeted agents.

Table 2. Univariate and multivariate analyses of progression-free survival.

Characteristics	7-y PFS (%)	Median PFS (y)	Univariate analysis			Multivariate analysis				Internal bootstrapping validation			
			95% CI	P	P*	HR	LCI	UCI	P	HR	LCI	UCI	Bootstrapping selection (%)
Binet A	40.3%	4.5	2.4-6.6	0.356	-	-	-	-	-	-	-	-	-
Binet B-C	30.0%	4.6	3.8-5.4										
<i>IGHV</i> mutated	49.3%	6.5	3.8-9.2	<0.001	0.003	-	-	-	0.001	-	-	-	98.8%
<i>IGHV</i> unmutated	23.0%	3.9	3.5-4.4			1.8	1.3	2.6		1.9	1.3	2.7	
No 11q deletion	33.4%	5.0	4.2-5.9	0.025	0.700	-	-	-	-	-	-	-	-
11q deletion	13.9%	3.6	2.4-4.9			-	-	-		-	-	-	
No 17p deletion	33.0%	4.8	4.1-5.6	<0.0001	<0.0001	-	-	-	<0.0001	-	-	-	99.5%
17p deletion	nr	1.1	0-2.6			4.0	2.2	7.5		4.9	2.5	9.8	
<i>TP53</i> wildtype	33.8%	5.4	4.3-5.8	<0.0001	<0.001	-	-	-	0.030	-	-	-	73.3%
<i>TP53</i> mutated	nr	2.8	2.0-3.5			1.7	1.1	2.8		1.8	1.1	3	
<i>BIRC3</i> wildtype	32.2%	4.8	4.1-5.6	<0.001	0.005	-	-	-	0.004	-	-	-	91.1%
<i>BIRC3</i> mutated	nr	2.2	0.9-3.5			2.8	1.4	5.6		3.4	1.6	7.3	
<i>EGR2</i> wildtype	31.5%	4.7	3.9-5.4	0.015	0.420	-	-	-	-	-	-	-	-
<i>EGR2</i> mutated	nr	1.5	0-3.8			-	-	-		-	-	-	
<i>ATM</i> wildtype	32.5%	4.8	4.1-5.6	0.029	0.812	-	-	-	-	-	-	-	-
<i>ATM</i> mutated	nr	3.2	2.4-4.1			-	-	-		-	-	-	

y: year; P: P-value; P*: Bonferroni correction; PFS: progression-free survival; CI: confidence interval; HR: hazard ratio; LCI: lower boundary of the confidence interval; UCI: upper boundary of the confidence interval; *IGHV*: immunoglobulin heavy variable gene; nr: not reached.

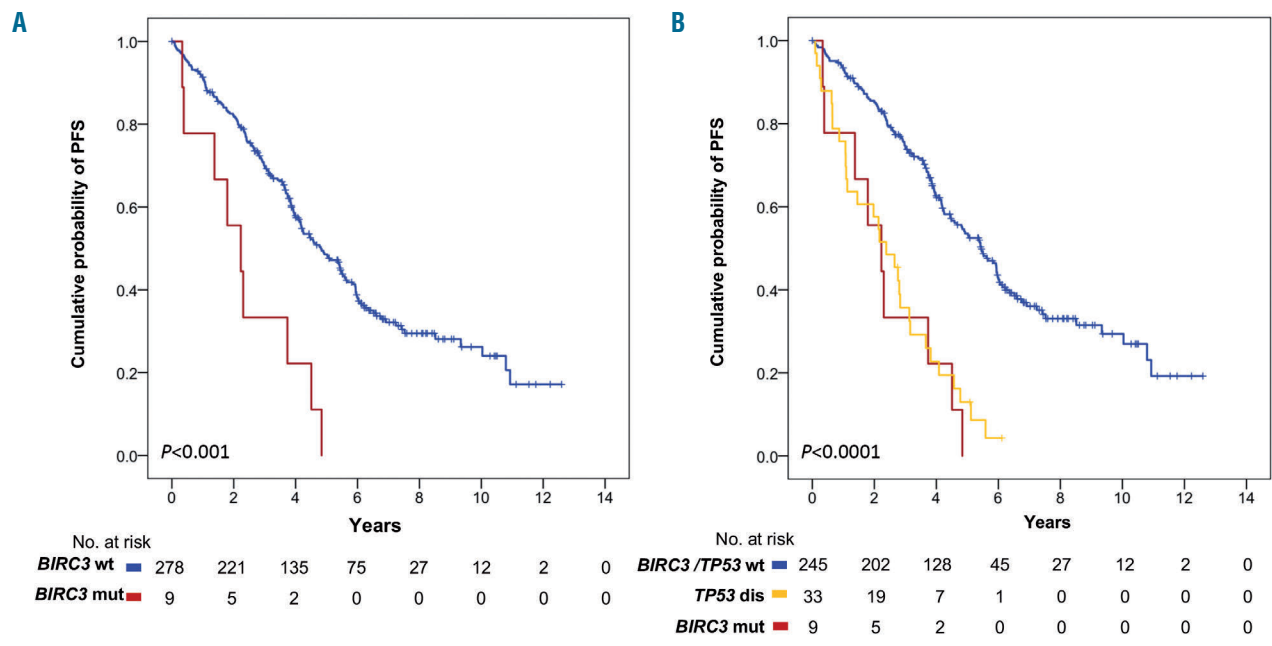


Figure 6. Kaplan-Meier estimates of progression-free survival in *BIRC3*-mutated patients. (A) Cases harboring *BIRC3* mutations are represented by the red line. *BIRC3*-wildtype cases are represented by the blue line. (B) Cases harboring *BIRC3* mutations are represented by the red line. Cases harboring *TP53* disruption (including *TP53* mutation and/or 17p deletion) are represented by the yellow line. Patients devoid of *BIRC3* mutation and *TP53* disruption are represented by the blue line.

Non-canonical NF- κ B activation by *BIRC3* mutations bypasses the block of BTK by ibrutinib. Consistently, NF- κ B activation and cell survival are unaffected by ibrutinib in both CLL cells (our study) and mantle cell lymphoma cells.¹⁴ If this preclinical evidence is validated in ibrutinib-treated patients, *BIRC3* mutations may also translate into a biomarker for informing selection of novel agents.

Acknowledgments

This work was supported by: *Molecular bases of disease dissemination in lymphoid malignancies to optimize curative therapeutic strategies, (5 x 1000 n. 21198), Associazione Italiana per la Ricerca sul Cancro Foundation, Milan, Italy (grants to GG and RF) and Progetti di Rilevante Interesse Nazionale (PRIN),*

(2015ZMRFEA), Rome, Italy; partially funded by the AGING Project – Department of Excellence – DIMET, Università del Piemonte Orientale, Novara, Italy; partially funded by Novara AIL ONLUS, Novara, Italy; Associazione Italiana per la Ricerca sul Cancro (AIRC IG-17314); Swiss Cancer League, ID HSR-4660-11-2018, Bern, Switzerland; Research Advisory Board of the Ente Ospedaliero Cantonale, Bellinzona, Switzerland; European Research Council (ERC) Consolidator grant CLL-CLONE ID: 772051; grant n. 320030_169670/1 Swiss National Science Foundation, Berne, Switzerland; Fondazione Fidinam, Lugano, Switzerland; Nelia & Amadeo Barletta Foundation, Lausanne, Switzerland; Fond'Action, Lausanne, Switzerland; Translational Research Program, grant n. 6594-20, The Leukemia & Lymphoma Society, New York, USA.

References

- Mansouri L, Papakonstantinou N, Ntoufa S, Stamatopoulos K, Rosenquist R. NF- κ B activation in chronic lymphocytic leukemia: a point of convergence of external triggers and intrinsic lesions. *Semin Cancer Biol.* 2016; 39:40-48.
- Bonizzi G, Karin M. The two NF- κ B activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* 2004;25(6):280-288.
- Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF- κ B signaling pathways. *Nat Immunol.* 2011;12(8):695-708.
- Sun SC. The noncanonical NF- κ B pathway. *Immunol Rev.* 2012;246(1):125-140.
- Asslaber D, Wacht N, Leisch M, Qi Y, et al. *BIRC3* expression predicts CLL progression and defines treatment sensitivity via enhanced NF- κ B nuclear translocation. *Clin Cancer Res.* 2019;25(6):1901-1912.
- Puente XS, Beà S, Valdés-Mas R, et al. Non-coding recurrent mutations in chronic lymphocytic leukaemia. *Nature.* 2015;526(7574):519-524.
- Landau DA, Tausch E, Taylor-Weiner AN, et al. Mutations driving CLL and their evolution in progression and relapse. *Nature.* 2015;526(7574):525-530.
- Hallek M, Cheson BD, Catovsky D, et al. Guidelines for diagnosis, indications for treatment, response assessment and supportive management of chronic lymphocytic leukemia. *Blood.* 2018;131(25):2745-2760.
- Gaidano G, Rossi D. The mutational landscape of chronic lymphocytic leukemia and its impact on prognosis and treatment. *Hematology Am Soc Hematol Educ Program.* 2017;2017(1):329-337.
- 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(16):1921-1924.
- 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(3):303-309.
- 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(2):208-215.
- Rossi D, Fangazio M, Rasi S, et al. Disruption of *BIRC3* associates with fludarabine chemorefractoriness in TP53 wild-type chronic lymphocytic leukemia. *Blood.* 2012;119(12):2854-2862.
- Rahal R, Frick M, Romero R, et al. Pharmacological and genomic profiling identifies NF- κ B-targeted treatment strategies for mantle cell lymphoma. *Nature Medicine.* 2014;20(1):87-92.
- Hallek M, Fischer K, Fingerle-Rowson G, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet.* 2010;376(9747):1164-1174.
- Stilgenbauer S, Schnaiter A, Paschka P, et al. Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. *Blood.* 2014;123(21):3247-3254.
- Grossmann V, Kohlmann A, Schnittger A, et al. Recurrent ATM and *BIRC3* mutations in patients with chronic lymphocytic leukemia (CLL) and deletion 11q22-q23. *Blood.* 2012;120(21):1771.
- Rose-Zerilli MJ, Forster J, Parker H, et al. ATM mutation rather than *BIRC3* deletion and/or mutation predicts reduced survival in 11q-deleted chronic lymphocytic leukemia: data from the UK LRF CLL4 trial. *Haematologica.* 2014;99(4):736-742.
- Baliakas P, Hadzidimitriou A, Sutton LA, et al. Recurrent mutations refine prognosis in chronic lymphocytic leukemia. *Leukemia.* 2015;29(2):329-336.
- Nadeu F, Delgado J, Royo C, et al. Clinical impact of clonal and subclonal TP53, SF3B1, *BIRC3*, NOTCH1, and ATM mutations in chronic lymphocytic leukemia. *Blood.* 2016;127(17):2122-2130.
- Vince JE, Wong WW, Khan N, et al. IAP antagonists target cIAP1 to induce TNF α -dependent apoptosis. *Cell.* 2007;131(4):682-693.
- Jost PJ, Ruland J. Aberrant NF- κ B signaling in lymphoma: mechanisms, consequences, and therapeutic implications. *Blood.* 2007;109(7):2700-2707.
- Raponi S, Del Giudice I, Ilari C, et al. Biallelic *BIRC3* inactivation in chronic lymphocytic leukaemia patients with 11q deletion identifies a subgroup with very aggressive disease. *Br J Haematol.* 2019;185(1):156-159.
- Hewamana S, Lin TT, Jenkins C, et al. The novel nuclear factor- κ B inhibitor LC-1 is equipotent in poor prognostic subsets of chronic lymphocytic leukemia and shows strong synergy with fludarabine. *Clin Cancer Res.* 2008;14(24):8102-8111.
- Hewamana S, Alghazal S, Lin TT, et al. The NF- κ B subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target. *Blood.* 2008;111:4681-4689.
- Beg AA, Baltimore D. An essential role for NF- κ B in preventing TNF- α -induced cell death. *Science.* 1996;274:782-784.
- Wang CY, Mayo MW, Baldwin AS, Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF- κ B. *Science.* 1996;274:784-787.
- Webster GA, Perkins ND. Transcriptional cross talk between NF- κ B and p53. *Mol Cell Biol.* 1999;19:3485-3495.
- Nakanishi C, Toi M. Nuclear factor- κ B inhibitors as sensitizers to anticancer drugs. *Nat Rev.* 2005;5:297-309.