Integration of B-cell receptor-induced ERK1/2 phosphorylation and mutations of SF3B1 gene refines prognosis in treatment-naive chronic lymphocytic leukemia

Signaling events downstream of the B-cell receptor (BCR) are key determinants of the clinical behavior of chronic lymphocytic leukemia (CLL). Extracellular signal-regulated kinase 1/2 (ERK1/2) is a major pathway downstream of BCR stimulation. In a recent study, applying a single cell network profiling (SCNP) assay to independent sets of CLL samples, we characterized functional elements of the BCR signaling network associated with clinical outcomes in CLL and validated BCR-induced ERK1/2 phosphorylation as a significant, independent predictor of faster clinical progression in CLL. Novel recurrent mutated genes have recently been associated with progressive or high-risk disease and a dismal outcome in CLL. Gene alterations occur during cancerous transformation and evolution of the malignant clone, most likely favored by signals from the BCR and microenvironment. Despite the established driving role of BCR signaling and gene mutations in CLL, little is known about the association between functional BCR responses and specific genetic alterations. Investigating the physiology of BCR signaling in the genetic context would add useful information to improve prognostic classification of CLL patients and gain deeper insights into pathobiological mechanisms. Herein, we investigated the impact of integrated BCR-induced ERK1/2 phosphorylation and recurrent gene mutations on prognosis of untreated CLL. We found that integrated dynamic ERK1/2 phosphorylation and SF3B1 mutations are associated with the risk of disease progression and enable identification of a novel intermediate-risk group of patients.

ERK1/2 phosphorylation (pERK1/2) was measured by SCNP in 152 treatment-naive CLL cell samples (Online Supplementary Table S1), in basal condition and following BCR stimulation with anti-IgM (Figure 1A,B). In the basal condition, we detected no differences in pERK1/2 between subgroups of patients defined by IGHV mutational status (UM: unmutated; M: mutated), CD38 expression, and cytogenetic mutations (n=141). Different sample sizes depend on availability of biological parameters. Data are expressed as equivalent number of reference fluorophores (ERF) and represented as Tukey boxes and whiskers. The Mann-Whitney test and Kruskal-Wallis test were used for comparisons. NS: not significant.

Figure 1. ERK1/2 phosphorylation in B cells from CLL patients. (A) Representative flow cytometry histograms of pERK1/2 in the basal condition or following BCR modulation with anti-IgM in CD5+/CD19+ cells from CLL patients, compared with autofluorescence signals. (B) pERK1/2 in CLL patients, in the basal condition and following anti-IgM modulation (n=152). Data are expressed as equivalent number of reference fluorophores (ERF). The results for basal and anti-IgM-modulated CLL cells were compared by the two-sample Wilcoxon signed rank sum test. The lines indicate the medians. The Y-axis is expanded on low values. (C-D) SCNP signals of pERK1/2 in the basal condition (C) and in response to anti-IgM stimulation (D) was associated with IGHV status (n=134), CD38 expression (n=152), and cytogenetic mutations (n=141). Different sample sizes depend on availability of biological parameters. Data are expressed as equivalent number of reference fluorophores (ERF) (C) and Uu (D) and represented as Tukey boxes and whiskers. The Mann-Whitney test and Kruskal-Wallis test were used for comparisons. NS: not significant.
TION AND CYTOGENETIC ALTERATIONS (FIGURE 1C). IN CONTRAST, pERK1/2 IN RESPONSE TO BCR STIMULATION (ANTI-lgM→ pERK1/2) WAS HIGHER IN UM-CLL THAN IN M-CLL AND HIGHER IN CD38-POSITIVE CASES THAN IN CD38-NEGATIVE CASES (FIGURE 1D). Moreover, higher anti-lgM→ pERK1/2 was associated with treatment requirement during follow-up (ONLINE SUPPLEMENTARY TABLE S2).

MUTATIONS OF NOTCH1, SF3B1, TP53, BIRC3, AND MYD88 GENES WERE EXAMINED IN CLL SAMPLES FOR WHICH DNA WAS AVAILABLE (146/152) BY POLYMERASE CHAIN REACTION AMPLIFICATION AND SANGER SEQUENCING (DETAILS IN ONLINE SUPPLEMENTARY DATA). DUE TO THE LOW NUMBER OF BIRC3 (N=2/146) AND MYD88 (N=2/146) MUTATIONS, THESE GENES WERE EXCLUDED FROM ANALYSIS.

NOTCH1 MUTATIONS WERE DETECTED IN 17/146 (12%) SAMPLES. OF THESE 17 PATIENTS, 15 (88%) CARRIED MISSENSE MUTATIONS AND 2/17 (12%) HAD NON-SENSE EVENTS: THESE LATTER WERE EXCLUDED FROM THE ANALYSIS. NOTCH1 MUTATIONS WERE ASSOCIATED WITH HIGHER CD38 EXPRESSION AND SHOWED A TRENDS TOWARD ASSOCIATION WITH TRISOMY 12 (FIGURE 2A; ONLINE SUPPLEMENTARY TABLE S3). NO ASSOCIATIONS WERE FOUND BETWEEN NOTCH1 MUTATIONS AND ANTI-lgM→ pERK1/2 (FIGURE 2A; ONLINE SUPPLEMENTARY TABLE S3).

SF3B1 GENETIC LESIONS WERE DETECTED IN 19/146 (13%) PATIENTS. THESE MUTATIONS WERE ASSOCIATED WITH UM-IGHV STATUS (FIGURE 2A; ONLINE SUPPLEMENTARY TABLE S3) AND MORE FREQUENTLY DETECTED IN ADVANCED (BINET STAGE B-C) STAGE DISEASE AND IN PATIENTS REQUIRING TREATMENT DURING FOLLOW-UP (ONLINE SUPPLEMENTARY TABLE S2). OF NOTE, CASES HARBOURING SF3B1 MUTATIONS ALSO EXHIBITED A HIGHER ANTI-lgM→ pERK1/2 (FIGURE 2A-B; ONLINE SUPPLEMENTARY TABLE S3). GIVEN THE STRONG ASSOCIATION BETWEEN ANTI-lgM→ pERK1/2 AND UM-IGHV STATUS (FIGURES 1D AND 2A), WE SOUGHT TO ASSESS THE INFLUENCE OF IGHV STATUS ON THE ASSOCIATION BETWEEN ANTI-lgM→ pERK1/2 AND SF3B1 MUTATIONS. SUPERIMPOSITION OF IGHV STATUS REVEALED THAT IN M-CLL SF3B1 MUTATIONS DID NOT IDENTIFY CLL SUBGROUPS WITH DIFFERENT ANTI-lgM→ pERK1/2 (FIGURE 2B). THEREFORE, IN M-CLL SF3B1 MUTATIONS APPEARED NOT TO HAVE A SIGNIFICANT IMPACT ON ANTI-lgM→ pERK1/2. IN UM-CLL, SF3B1-MUTATED CASES EXHIBITED A HIGHER ANTI-lgM→ pERK1/2 THAN WILD-TYPE (WT) SF3B1 CASES (FIGURE 2B). THE MORE FREQUENT ASSOCIATION OF UM-IGHV WITH ERK1/2 RESPONSIVENESS TO BCR IS, THEREFORE, MAINLY EXPLAINED BY UM-CLL SAMPLES THAT HARBOR SF3B1 MUTATIONS.

TP53 GENE MUTATIONS WERE DETECTED IN 11/146 (8%) CELL SAMPLES, WITH ONE PATIENT HARBORING TWO MUTATIONS. AS EXPECTED, TP53 LESIONS WERE ENRICHED IN PATIENTS WITH DEL(17P) (FIGURE 2A; ONLINE SUPPLEMENTARY TABLE S3). WHILE CASES WITH TP53 LESIONS EXHIBITED A TREND TOWARD ASSOCIATION WITH HIGH ANTI-lgM→ pERK1/2, CASES WITH p53 DYSFUNCTION (TP53 LESIONS AND/OR DEL(17P)) SHOWED NO ASSOCIATION WITH HIGH ANTI-lgM→ pERK1/2 (FIGURE 2A; ONLINE SUPPLEMENTARY TABLE S3).

CONCURRENT MUTATIONS WERE DETECTED IN ONLY TWO CASES (COEXISTING NOTCH1 AND TP53 MUTATIONS) AND THEIR ASSOCIATION WITH pERK1/2 WAS NOT, THEREFORE, INVESTIGATED.

TO ASSESS THE IMPACT OF pERK1/2 INTEGRATED WITH GENE MUTATIONS ON DISEASE PROGRESSION, AS MEASURED AS TIME TO FIRST TREATMENT (TTFT), WE EXAMINED TIME-TO-EVENT MODELING UTILIZING ERK1/2 SCNP DATA, RECURRENT GENE MUTATIONS, IGHV STATUS, CD38 EXPRESSION, AND CYTOGENETIC ALTERATIONS – ALONE AND IN COMBINATION – IN THE WHOLE SET OF PATIENTS FOR WHOM ALL STUDIED BIOLOGICAL AND TTFT DATA WERE AVAILABLE (N=125/152). UNIVARIATE TIME-TO-EVENT ANALYSIS IDENTIFIED HIGHER ANTI-lgM→ pERK1/2 AS A SIGNIFICANT, INDEPENDENT PREDICTOR OF SHORTER TTFT. WHILE HIGH ANTI-lgM→ pERK1/2 WAS NOT ASSOCIATED WITH SHORTER TTFT IN THE COMPREHENSIVE MULTIVARIATE ANALYSIS, IN A BIVARIATE TIME-TO-EVENT ANALYSIS HIGH ANTI-lgM→ pERK1/2 AND SF3B1 MUTATION WERE FOUND TO BE INDEPENDENT, SIGNIFICANT PARAMETERS OF PROGNOSIS (ONLINE SUPPLEMENTARY TABLE S4A). REMARKABLY, INTEGRATING ANTI-lgM→ pERK1/2 DATA AND SF3B1 MUTATION, THREE INDEPENDENT PROGNOSTIC CATEGORIES WERE IDENTIFIED: A LOW-RISK GROUP (N=42; PATIENTS REQUIRING TREATMENT=26%; MEDIAN TTFT=40 MONTHS) COMPRISED PATIENTS WITH LOW ANTI-lgM→ pERK1/2 AND WT SF3B1; AN INTERMEDIATE-RISK
group (n=66; patients requiring treatment=48%; median TTFT=34 months) included patients with low anti-IgM→pERK1/2 and mutated SF3B1 or high anti-IgM→pERK1/2 and wt SF3B1; and a high-risk group (n=17; patients requiring treatment=82%, median TTFT=3 months) comprised patients with high anti-IgM→pERK1/2 and mutated SF3B1 (Figure 3C).

A subset of Binet stage A patients for whom all biological and TTFT data were available (n=90/112) was then analyzed. Univariate time-to-event analysis identified increased anti-IgM→pERK1/2 (Figure 3D), mutated SF3B1 (Figure 3E), mutated TP53 (P=0.001), UM-IGHV (P<0.0001), and CD38-positivity (P=0.0006) as significant, independent predictors of shorter TTFT. The bivariate time-to-event analysis identified high anti-IgM→pERK1/2 and SF3B1 mutation as independent significant variables in terms of TTFT (Supplementary Table S4B). Integrating anti-IgM→pERK1/2 and SF3B1 mutation, we could stratify early-stage patients into three independent prognostic categories: a low-risk group (n=33; patients requiring treatment=18%, median TTFT=47 months) comprised patients with low anti-IgM→pERK1/2 and wt SF3B1; an intermediate-risk group (n=49; patients requiring treatment=37%, median TTFT=42 months) included patients with low anti-IgM→pERK1/2 and mutated SF3B1 or high anti-IgM→p-ERK1/2 and wt SF3B1; and a high-risk group (n=8; patients requiring treatment =75%, median TTFT=16 months) comprised patients with high anti-IgM→pERK1/2 and mutated SF3B1 (Figure 3F).

In this study, we measured ERK1/2 response to BCR in association with NOTCH1, SF3B1, TP53 mutations with the aim of integrating, for the first time, dynamic properties of BCR activation and driver-gene mutations in a prognostic model for treatment-naïve CLL. Our data reveal that integrating dynamic ERK1/2 phosphorylation and SF3B1 mutational status improves the prognostic potential of pERK1/2 and SF3B1 alone and identifies three groups of patients with distinct risks of disease progression.

The finding that high anti-IgM→pERK1/2 identifies a subgroup of CLL treatment-naïve patients with a more aggressive disease and a faster clinical progression confirms our previous data obtained from patients with early-stage disease and extend them to patients with advanced-stage disease. Overall, these data identify ERK1/2 as a relevant node on the route of BCR signaling, which is able to capture the behavior of other components of the pathway. In line with this, ERK1/2 functions as a survival pathway and is required for cell cycle progression in CLL. Of note, a recent study showed that novel CLL driver mutations affect the ERK pathway, with 8.7% of patients carrying at

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**Figure 3. Kaplan-Meier curves of time to first treatment (TTFT).** Kaplan-Meier curves of TTFT for the entire patient set for which biological parameters and clinical data were available (n=125/152, A-C) or for the Binet stage A patients for whom biological parameters and clinical data were available (n=90/112, D-F). Curves were defined by pERK1/2 response to BCR (anti-IgM→pERK1/2), using the pre-specified 0.66 Uu cut-off (A,D), the presence of SF3B1 mutations (B,E), or the integrated anti-IgM→pERK1/2 data (cut-off=0.66) and SF3B1 mutations (C,F).
least one gene mutation in this pathway.11 Interestingly, the response of ERK1/2 to BCR, rather than its basal phosphorylation state, is associated with standard prognostic parameters (UM-IGHV and CD38 positivity, but not cytogenetic alterations) and disease progression, supporting the determinant role of dynamic properties of BCR signaling in CLL. These data are in agreement with a recent study by D’Avola et al. showing that BCR responsiveness, measured as percentage of intracellular Ca2+ mobilization, is associated with UM-IGHV and CLL aggressiveness.3 In contrast with our results, that study showed an association between BCR responsiveness and trisomy 12 and del(17p). These discrepancies might be due to differences in sets of patients and measurements of BCR response.

To measure ERK1/2 signaling in this study, we used SCNP, a phospho-specific assay based on flow cytometry, which provided ERK1/2 readout metrics for developing a mathematical model for the prediction of CLL progression.3,8 Overall, this study confirms that SCNP can capture biologically and clinically relevant information on pathological cells.

As expected, mutations of NOTCH1, SF3B1 and TP53 genes were more frequently detected in patients with unfavorable clinico-biological features.1,12 This was specifically pronounced for SF3B1, whose mutations were enriched in UM-CLL, advanced disease, and patients requiring treatment during follow-up. SF3B1 mutations were also associated with a higher response of ERK1/2 to BCR, specifically in UM-CLL. In contrast, D’Avola et al. revealed differential BCR responses between wt and mutated SF3B1 in M-CLL but not in UM-CLL. Besides differences in the BCR-response measurements, this discrepancy may be due to the low number of cases with SF3B1 mutations (7 in the study by D’Avola et al. and 3 in ours). Further studies with larger series of patients are required to address this issue.

Only SF3B1 mutations integrated with anti-IgM→pERK1/2 can refine prognosis in CLL, which might suggest a direct effect of SF3B1 mutations on BCR signaling. SF3B1 is a core component of the RNA splicing machinery14 and, among other effects, SF3B1 mutations might induce alternative splicing of the BCR component CD79b. A change in the balance between the entire CD79b transcript and the alternative spliced form could account for altered BCR expression and function in CLL.15

In conclusion, this study shows that integrated ERK1/2 response to BCR stimulation and SF3B1 gene mutations refine prognosis in CLL and may form the basis for future investigations aimed at validating an integrated prognostic model including dynamic properties of BCR and SF3B1 mutation in CLL.

Chiara Cavallini,1 Carlo Visco,2 Santosh Putta,3 Davide Rossi,4 Elda Mioni,1 Norman Purvis,6 Ornellia Lovato,1 Omar Perbellini,4 Erika Falisi,1 Monica Fatoco,2 Livio Trentin,7 Maria G. Romanelli,7 Gianpietro Semenzato,7 Achille Ambrosotti,7 Gianluca Gaidano,8 Giovanni Pizzolo,8 Alessandra Cesano5,6 and Maria T. Scupoli1,5

1 Research Center LURM, University of Padua, Italy; 2 Department of Cell Therapy and Hematology, San Bortolo Hospital, Vicenza, Italy; 3 Qognit Inc., Foster City, CA, USA; 4 Hematology, Oncology Institute of Southern Switzerland and Institute of Oncology Research, Bellinzona, Switzerland; 5 Department of Medicine, Section of Hematology, University of Verona, Italy; 6 Pierian Biosciences, Franklin, TN, USA; 7 Department of Medicine, Hematology and Clinical Immunology Branch, University of Padua, Italy; 8 Department of Neurosciences, Biomedicine and Movement Sciences, Section of Biology and Genetics, University of Verona, Italy; 9 Department of Translational Medicine, Division of Hematology, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy and 10 NanoString, Inc, Seattle, WA, USA

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Correspondence: mariaturesa.scupolo@univr.it

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