Over-Time Monitoring of CXCR4dim/CD5bright Proliferative Fraction Anticipates Relapse In Ibrutinib-Treated CLL : a reservoir for BTK mutations?

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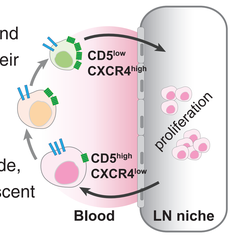
Federico Pozzo[1], Gabriela Forestieri[2], Filippo Vit[1], Erika Tissino[1], Tamara Bittolo[1], Giulia Ianna[1], Lodovico Terzi di Bergamo[2], Roberta Laureana[3], Agostino Tafuri[4], Annalisa Chiarenza[5], Francesco Di Raimondo[5], Jacopo Olivieri[6], Francesco Zaja[7], Luca Laurenti[8], Maria Ilaria del Principe[3], Riccardo Bomben[1], Antonella Zucchetto[1], Davide Rossi[2], Valter Gattei[1]

1 Clinical and Experimental Onco-Hematology Unit, Centro di Riferimento Oncologico di Aviano (CRO), IRCCS, Aviano (PN), Italy; 2 Experimental Hematology, Institute of Oncology Research, Bellinzona, Switzerland; 3 Hematology, Dept of Biomedicine and Prevention, University Tor Vergata, Rome, Italy; 4 Hematology Unit, Azienda Ospedaliera-Universitaria Sant'Andrea, Rome, Italy; 5 Division of Haematology, Ferrarotto Hospital, Catania, Italy; 6 Hematology Clinic, ASUFC, Udine, Italy; 7 Department of Medical, Surgical and Health Sciences, University of Trieste, Italy; 8 Institute of Hematology, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome, Italy.

Background

BTK inhibitors deeply affect the proliferative capacity, homing and migrations of chronic lymphocytic leukemia (CLL) cells, and cause their redistribution from nodal compartments to the blood stream. Reciprocal expression of CXCR4 and CD5 on circulating CLL cells is an established marker discriminating the proliferative fraction (CD5high/CXCR4dim, PF), recently egressed from the lymph node, from the resting fraction (CXCR4high/CD5dim, RF) of older, quiescent cells navigating the blood stream.



Adapted from Bartholdy et al., Blood Adv. 2022

Aim

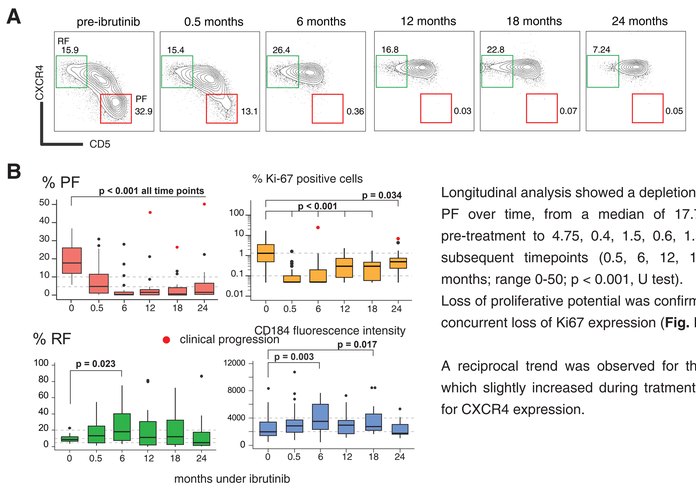
Here we investigate the dynamics of the CD5bright/CXCR4dim proliferative fraction under long-term IBR treatment and its correlation with emerging resistance mutations of BTK/PLCG2 and with clinical relapse.

Methods

Peripheral blood (PB) samples were collected from CLL patients who either entered the IOSI-EMA-001 study (NCT02827617; n=31) or were referred to our institution for routine immunogenetic analyses (n=101). Immunophenotypic analyses and cell sortings were performed using a FACSCanto II and a FACSAriaIII flow cytometer/cell sorter (BD Bioscience). DNA sequencing was performed with a targeted amplicon strategy, while RNA-seq was performed with the mRNA Library prep Kit on a Nextseq instrument (Illumina) and analyzed with R package DESeq2 and GSEA. Groups were compared with Mann-Whitney rank-sum test; significant p-values ($p<0.05$) are reported.

Results: PF is suppressed under ibrutinib

We monitored the dynamics of the PF under the course of IBR therapy in 31 CLL cases recruited in the IOSI-EMA-001 study (NCT02827617) with sequential blood sampling (156 samples, median 6 samples/case, range 3-7). PF was monitored through fixed gates, designed on the pre-treatment sample around the around the CXCR4dim/CD5bright populations (**Fig. A**).

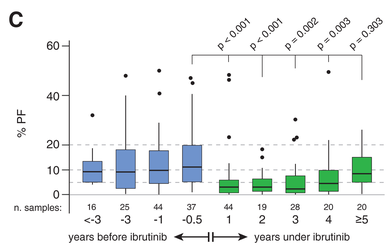


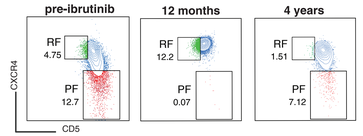
Longitudinal analysis showed a depletion of the PF over time, from a median of 17.7% at pre-treatment to 4.75, 0.4, 1.5, 0.6, 1.5% at subsequent timepoints (0.5, 6, 12, 18, 24 months; range 0-50; $p < 0.001$, U test).
Loss of proliferative potential was confirmed by concurrent loss of Ki67 expression (**Fig. B**).

A reciprocal trend was observed for the RF, which slightly increased during tratment, and for CXCR4 expression.

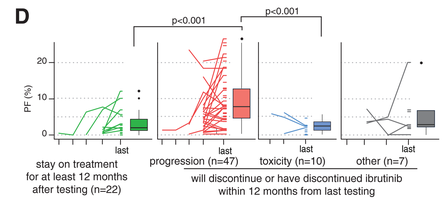
Results: PF dynamics under ibrutinib

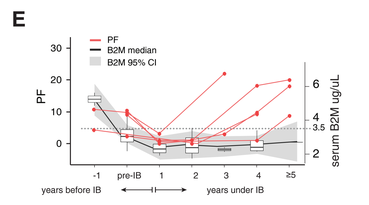
We retrospectively analyzed the PF from a second cohort of 101 ibrutinib-treated CLL cases from the real world, referred for routine immunophenotyping (291 samples, median 3 samples/case, range 3-7). Median PF of pre-IBR samples was 11.0% (range 0.7-47%), dropping to ~2% within 2 years of treatment.
However, at 4 and 5 years, we observed an apparent rebound of the PF , with a median increase at 4 years to 4.9% and to 8.2% at five or more years (**Fig. C**).



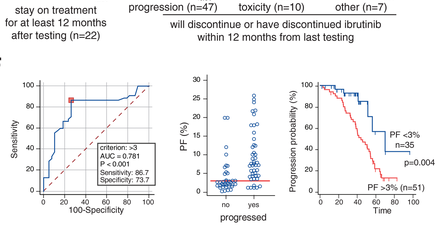


To correlate the reappearance of the PF with the cause of IBR discontinuation, we focused on 64 samples collected within 12 months before/after IBR discontinuation due to progression (n=47), toxicity (n=10) or other/death (n=7), and compared with 22 samples still on treatment for the next 12 months after testing.
Cases who will discontinue IBR within 1-12 months from/after testing because of relapse presented a significantly higher PF compared to cases who cases who have not discontinued IBR for the following 12 months after testing (median 8.4 vs. 1.6; **Fig. D**) or cases who discontinued because of toxicity (median 8.4 vs. 2.2).



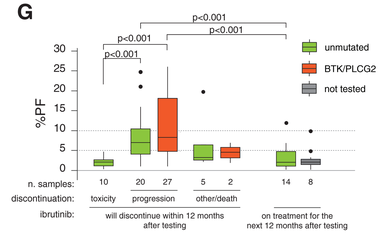


Sequential dosages of plasmatic B2M in progressing cases (n=5) revealed steady levels (median 2.38 ug/mL) below the 3.5 ug/mL threshold, while the PF increased (median PF 18%; **Fig.E**) until progression.



ROC analysis of the PF versus risk of progression selected a criterion of PF>3% as the most discriminating for a higher risk of progression (**Fig. F**), also significantly separating patients into two categories with different risk of progression (median 56 vs. 45 months, p=0.031).

Mutations of BTK/PLCG2 were detected in 31/101 cases; of these, 29 (93%) discontinued IBR due to progression/relapse and two cases because of other reason/death. Median time of appearance of BTK/PLCG2 mutations was 48 months (range 16-70). Median VAF for BTK was 22.8% (range 1.2-99.0) and 3.5% (range 1.7-17.6) for PLCG2.

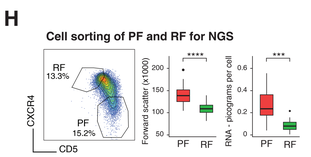


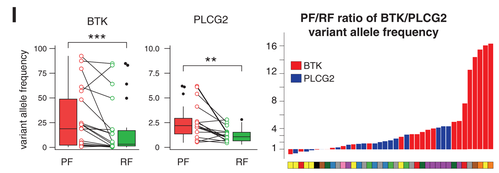
We stratified by presence of either mutation the 64 near-discontinuation samples; mutations were present in 27/47 progressing cases, with a PF higher than wild-type (WT) cases discontinuing for toxicity (8.4% vs. 2.2%) or still on treatment (8.4 vs. 2.1%; **Fig.G** upper comparisons)

Notably, the 20 WT progressing cases also showed higher PF compared to cases discontinuing for toxicity (6.4% vs. 2.2%) or to cases still on treatment (6.4% vs. 2.1%; **Fig. G** lower comparisons).

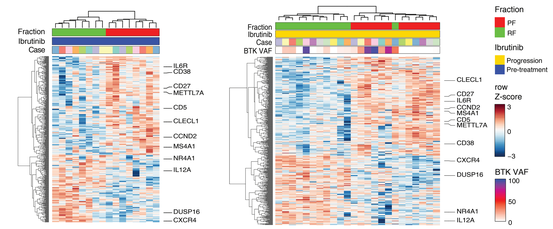
Results: ibrutinib-resistant PF at relapse

We isolated the PF and RF fractions from 10 relapsed cases with BTK/PLCG2 mutations, characterized by reappearance of the PF after prolonged IBR treatment (median 50.7 months); PF cells also showed larger size and greater RNA content (**Fig. H**). Sequencing revealed a mean of 1.7 BTK mutations per sample (range 1-4). Median VAF was higher in the PF (23.0%, range 0.3-92.3) than in the RF (5.15%, range 0.1-84.8; **Fig. I**). Overall, VAF in the PF was almost 3 times larger than the RF (average 2.82, range 0.01-38.0; **Fig. I**). Accompanying PLCG2 mutations were present in 6 cases, generally more frequent (average 3.7 mutations per case, range 1-8) but with lower VAF (median 1.7%, range 0.1-4.3); again, on average the PF fraction had higher VAF than the RF (average PF: 2.0, range 0.1-6.2; average RF: 1.35, range 0.4-2.8)

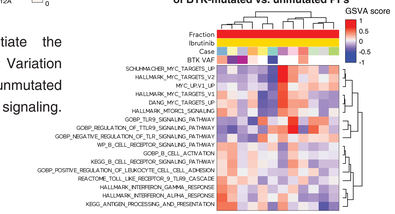




Finally, we performed mRNA-seq on PF/RF fractions from 12 cases, isolated at both pre-treatment and at progression. At pre-treatment, a differential expression signature of 479 genes clearly separated PF vs. RF (**Fig. J**); at progression, this signature drove co-clustering of 12 out of 13 fractions with the respective pre-treatment counterparts (**Fig. K**), confirming the functional similarity of the two PF fractions. Several published proliferation-related signatures (e.g. Calissano et al. Mol Med 2011, Bartholdy et al. Blood Adv. 2020, Seda et al. Blood 2021) were strongly enriched in pre-treatment samples but remained also significantly enriched at progression, suggesting that these transcriptional programs were comparably active (**Fig. L**).



We evaluated if different transcriptional programs could differentiate the BTK-unmutated from BTK-mutated PF fractions at progression. Gene Set Variation Analysis (GSVA) suggests a preferential usage of MYC signaling by BTK-unmutated PF fractions (**Fig. M**, upper-right quadrant), possibly through TLR9 signaling. Oppositely, BTK-mutated PF fractions, showed an enrichment of BCR-related gene sets, in agreement with a recovery of BTK functionality and a reactivation of the BCR pathway (**Fig. M**, lower-left quadrant).



Conclusions

- CXCR4dim/CD5bright PF population is efficiently suppressed by IBR but often reappears at relapse;
- The relapsed PF is associated with BTK/PLCG2 mutations, suggesting that these mutations occur within the lymph nodes, the proliferative compartment of CLL;
- The relapsed PF is functionally and phenotypically similar to the pre-Ibrutinib PF, and associated with MYC signaling in BTK-unmutates cases;
- Clinically, longitudinal monitoring of the PF may provide a simple tool helping to intercept CLL progression under IBR therapy.

AUTHORS DECLARE NO CONFLICT OF INTEREST