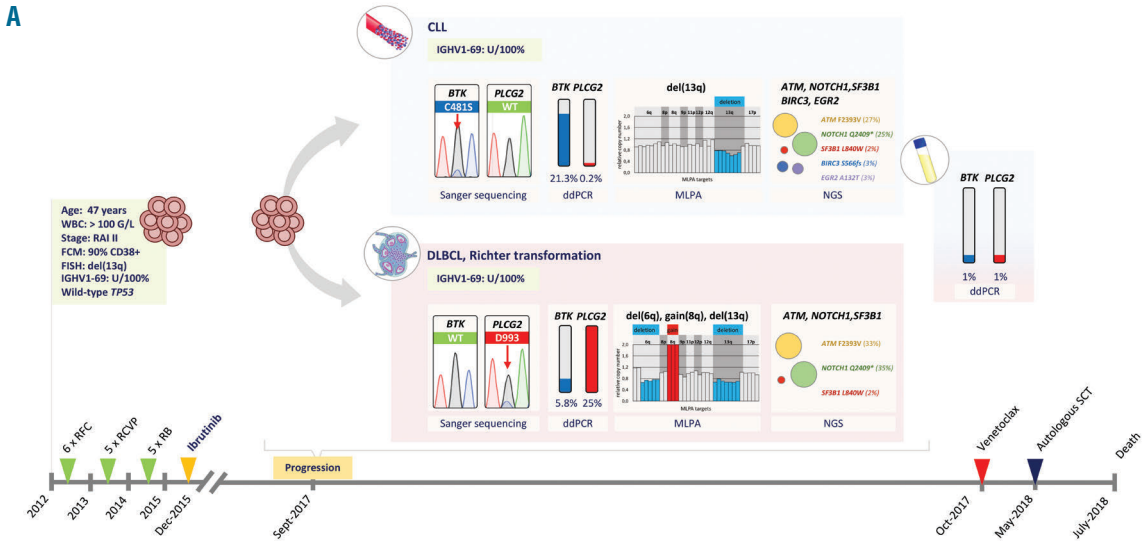
Spatial clonal evolution leading to ibrutinib resistance and disease progression in chronic lymphocytic leukemia

Ibrutinib has dramatically altered the therapeutic landscape of chronic lymphocytic leukemia (CLL) with remarkable responses in relapsed or refractory as well as in previously untreated CLL harboring *TP53* aberrations.[1,2] Despite durable responses in the majority of cases, approximately 20% of patients experience disease progression initiated by secondary therapy resistance. In the majority of patients progressing on ibrutinib, Bruton tyrosine kinase (*BTK*) or phospholipase *Cg2* (*PLCG2*) resistance mutations predate the manifestation of clinical progression by up to 15 months,[3] making early and sensitive detection of resistance mutations of major clinical importance. Longitudinal studies including CLL patients undergoing Richter transformation have shed some light on the temporal aspects of clonal evolution processes



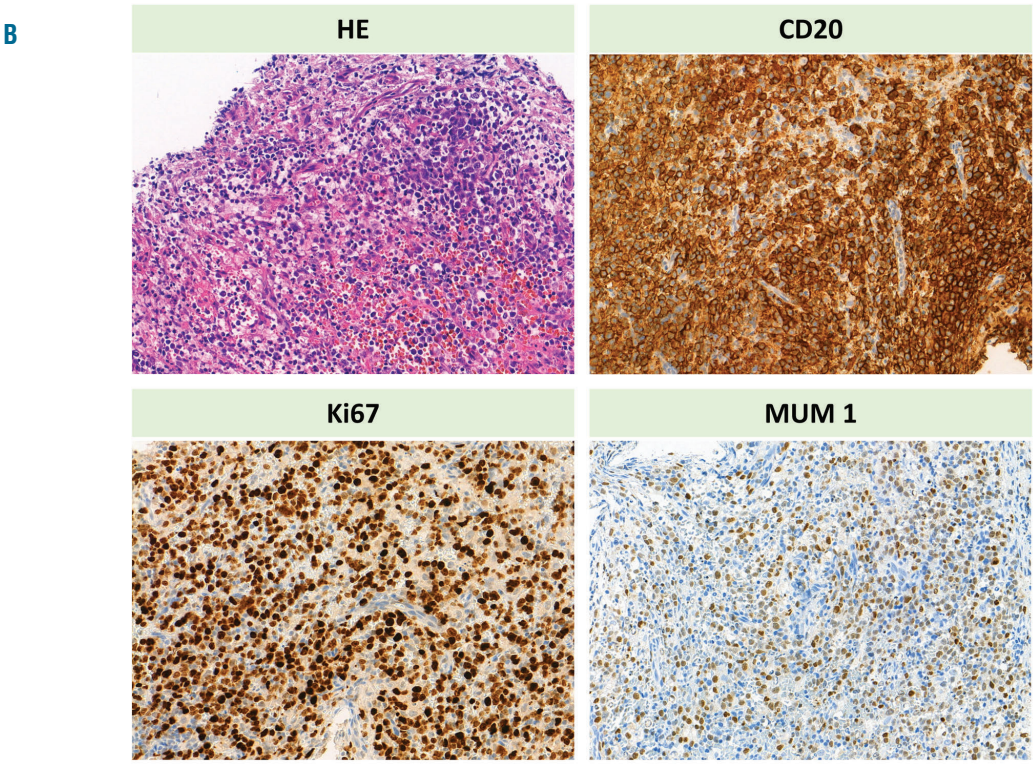
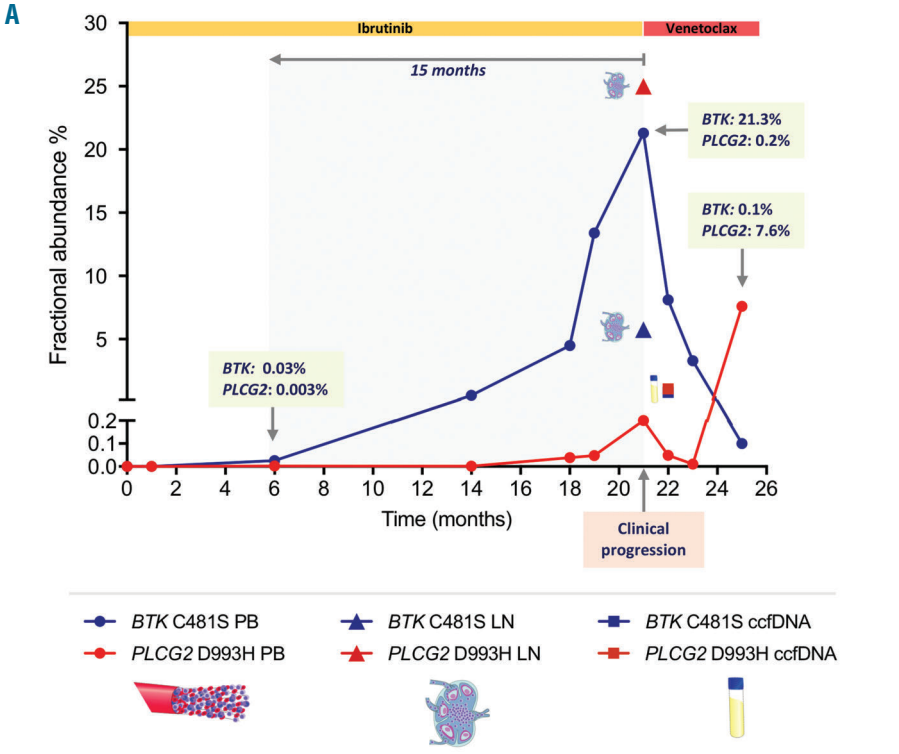


Figure 1. Detailed illustration of the clinical and genetic events at ibrutinib relapse and histological features of the transformed disease. (A) Illustrated are the main clinical and genetic events with a focus on the genetic heterogeneity observed at the time of ibrutinib relapse. (B) Morphological features and immunophenotype of the transformed diffuse large B-cell lymphoma. The images show hematoxylin and eosin, CD20, Ki67 and MUM1 stain (objective: 20x). ddPCR: digital droplet PCR; FCM: flow-cytometry; FISH: Fluorescence *in situ* hybridization; MLPA: Multiplex ligation-dependent probe amplification; RFC: rituximab-fludarabine-cyclophosphamid; RCVP: rituximab-cylcophosphamide-vincristine-prednisolone; RB: rituximab-bendamustine; SCT: stem cell transplantation.

leading to ibrutinib resistance,[4-6] however, the spatial heterogeneity with mutations residing in various environmental niches has not yet been reported in detail. Here, we dissect an example of a CLL patient treated with ibrutinib displaying spatial heterogeneity in terms of the ibrutinib resistance mutations, and we are able to document, for the first time, an example of ibrutinib-driven spatial convergent evolution leading to disease progression and transformation.

A 47-year-old man was diagnosed with a RAI stage II CLL with unmutated IGHV gene segment and 13q deletion in 90% of peripheral blood cells in August 2012. With an active and bulky disease, the patient required treatment and, starting in September 2012, he underwent three lines of chemoimmunotherapy (6 cycles of rituximab-fludarabine-cyclophosphamide (RFC), 5 cycles of rituximab-cylcophosphamide-vincristine-prednisolone (RCVP) and 5 cycles of rituximab-bendamustine (RB)), with the last cycle of RB administered in August 2015. The FCR treatment resulted in complete remission (CR), with partial remission achieved in response to both RCVP and RB regimens. The relapsed/refractory disease was subsequently treated with ibrutinib monotherapy (420 mg/day). Del(17p) and *TP53* mutation analyses were performed before all lines of therapies with negative results. The patient displayed a stable partial



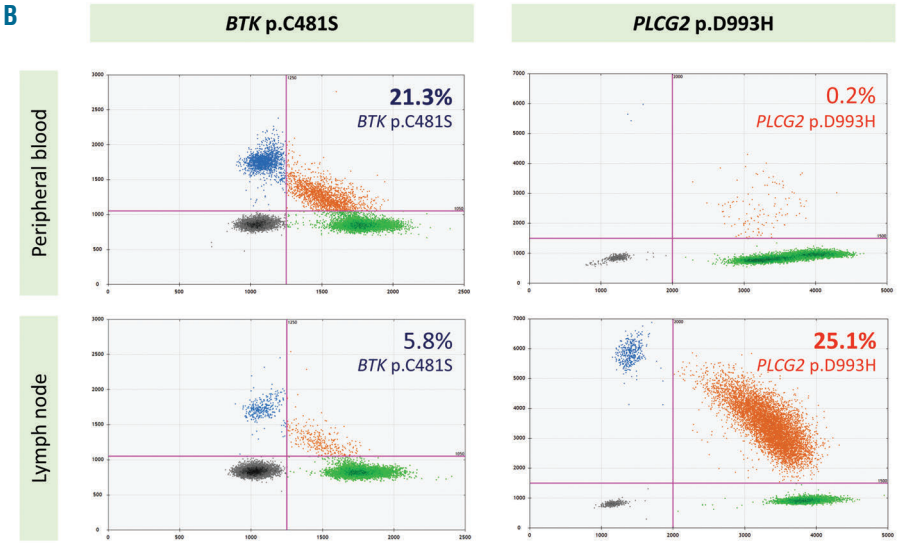


Figure 2. Spatiotemporal dynamics of the *BTK* and *PLCG2* mutations during ibrutinib treatment. (A) Illustrated are the fractional abundances of the *BTK* p.C481S and *PLCG2* p.D993H mutations in peripheral blood, lymph node and circulating cell-free DNA (ccfDNA) during the course of the disease, as defined by digital droplet PCR (ddPCR) assays. (B) Representative ddPCR plots illustrating the different representation of the *BTK* p.C481S and *PLCG2* p.D993H mutations in peripheral blood and lymph node. The mutation burden was expressed as fractional abundance (FA) with the FA calculated as the ratio between the number of mutant DNA molecules and the number of mutant plus wild type molecules. The FA values were normalized to the CLL cell content as defined by flow cytometry. ccfDNA: circulating cell-free DNA.

response on ibrutinib for 21 months until the emergence of simultaneous lymphadenomegaly and lymphocytosis with ibrutinib resistance. Core biopsy was taken from an enlarged lymph node, and histology examination revealed Richter transformation to diffuse large B-cell lymphoma (DLBCL) (Figure 1A). The clonally related lymphoma was positive for CD20, BCL2, MUM1 and c-MYC, and negative for CD10, CD30 and BCL6, with a Ki67 index of 70% (Figure 1B). In addition to a common 13q deletion, 6q deletion and gain 8q were identified exclusively in the lymph node by multiplex ligation-dependent probe amplification (Figure 1A). The patient was subsequently treated with venetoclax (ramping dosage from 20 mg to 400 mg daily within 5 weeks). Due to progression after three months, high-dose salvage chemotherapy was administered (2 cycles of R-ESHAP: rituximab + etoposide, cytarabine, cisplatin and methylprednisolone), followed by an autologous stem cell transplantation. The patient achieved complete remission assessed by CT scan and laboratory tests, but after a short, uneventful post-transplantational period, he relapsed and died on day 66 after transplantation, in July 2018.

To dissect the dynamics of mechanisms leading to spatial heterogeneity and ultimately conferring ibrutinib resistance, we analysed serial samples collected at ten different timepoints during the complete disease course (Figure 2A). Genomic DNA was isolated from ficoll-separated peripheral blood mononuclear cells and native lymph node tissue using the Qiagen AllPrep kit (Qiagen). Circulating cell-free DNA (ccfDNA) was isolated with QIAmp Circulating Nucleic Acid Kit (Qiagen) from peripheral blood samples collected in PAXgene Blood ccfDNA tubes (Qiagen). The fraction of tumor cells ($CD5^+/CD19^+$ cells) was determined by flow cytometry. Bidirectional Sanger sequencing of *BTK* exons 11, 15, 16, and *PLCG2* exons 12, 19, 20, 24, 27 and 30 was performed on an ABI3500 genetic analyser (ThermoFisher). Targeted ultra-deep next-generation sequencing (NGS) analysis covering the whole coding regions of the *BTK* and *PLCG2* genes was performed using a TruSeq Custom Amplicon approach on a MiSeq platform (Illumina). The same approach was used to perform NGS analysis of the hotspots of A*TM, BCOR, BIRC3, EGR2, NOTCH1, NFKBIE, MYD88, SF3B1* and *TP53* genes. Abundances of the *PLCG2* p.P993H and *BTK* p.C481S variants were quantified using custom assays on a QX200 droplet digital PCR system (ddPCR, BioRad). The IGHV mutation status was determined according the most recent European Research Initiative on CLL (ERIC) recommendation.[7] The study was conducted in accordance with the Declaration of Helsinki.

The clonal relationship between the CLL at diagnosis, at the time of ibrutinib resistance and following Richter transformation was confirmed by identifying an identical *IGH* gene rearrangement and unmutated IGHV1-69 gene segment in all three specimens analysed.

Sanger sequencing revealed a canonical *BTK* p.C481S mutation in the peripheral blood at the time of relapse following ibrutinib treatment. Intriguingly, a *PLCG2* p.D993H mutation was detected in the lymph node specimen, with the absence of the *BTK* p.C481S mutation present in peripheral blood, suggesting convergent evolution in terms of the *BTK* and *PLCG2* variants with both commonly associated with ibrutinib resistance. To further scrutinize this spatial heterogeneity, *BTK* and *PLCG2* mutations were screened by ultra-deep NGS which confirmed the Sanger sequencing data and, in addition to the dominant *PLCG2* p.D993H variant, identified the *BTK* p.C481S mutation as a minor clone in the lymph node, with the *BTK* p.C481S mutation remaining the exclusive variant in the peripheral blood (Figure 1A and 2A).

Next, we tested the presence of these mutations in the ccfDNA isolated at the time of ibrutinib relapse using a highly sensitive ddPCR approach. Notably, both mutations were identified in the ccfDNA with a VAF of 1% (Figure 2A), supporting previous findings that ccfDNA may well represent a reliable source for screening BTK/PLCG2 mutations in patients with CLL undergoing targeted therapy.[8]

The scrutiny of clonal heterogeneity observed between the peripheral blood and lymph node compartment was extended to the most frequent mutation targets known in CLL by performing an ultra-deep NGS analysis of the *ATM, BCOR, BIRC3, EGR2, NOTCH1, NFKBIE, MYD88, SF3B1* and *TP53* genes. In addition to the common *ATM* p.Phe2393Val, *NOTCH1* p.Gln2409* and *SF3B1* p.Leu840Trp mutations identified in both compartments, the *BIRC3* p.Ser566fs and *EGR2* p.Ala132Thr mutations were exclusively identified in the peripheral blood (Figure 1A). The other genes showed a wild-type genotype.

Recent studies suggest that driver mutations in CLL most likely emerge at a pre-leukemic multipotent hematopoietic progenitor stage, with at least a fraction of somatic mutations occurring before disease onset.[9,10] The spatial clonal heterogeneity may thus develop from a pre-existing diverse mutation repertoire by differential selective pressure of the treatment and microenvironmental effects in different anatomical niches. Although none of the previous studies detected *BTK* or *PLCG2* mutations prior to ibrutinib therapy, *in silico* analyses suggest that mutations even in these two genes may exist as early genetic alterations within a diverse cell population.[4,11]

To dissect the temporal and spatial dynamics of BTK and *PLCG2* mutant subclones, ddPCR analysis was performed on ten sequential peripheral blood samples collected during the course of the disease and a lymph node specimen obtained at the time of relapse, as summarized in Figures 1A and 2A. The *BTK* and *PLCG2* variants were absent in the pre-ibrutinib peripheral blood sample, however, their emergence predated the clinical progression by 15 months, with the *BTK* and *PLCG2* mutations becoming detectable at month 6 on ibrutinib therapy with minor VAFs of 0.03% and 0.003%, respectively (Figure 2A). We observed a gradual clonal expansion of these variants in the sequential samples, demonstrating an explicit clonal selection under the selective pressure of the treatment. Of note, the ddPCR assay identified the *PLCG2* p.D993H mutation as a minor clone (VAF: 0.2%), previously unseen in the peripheral blood sample by NGS at the time of ibrutinib relapse. At this timepoint, the *PLCG2* p.D993H mutation represented a major clone (VAF: 25%) in the lymph node sample, with a concurrent minor *BTK* p.C481S clone (VAF: 5.8%) (Figure 2A). The two subclones displayed differential sensitivity to the subsequent venetoclax therapy, most likely conferred by Richter transformation present in the lymph node compartment. In the peripheral blood, we observed a reduction of the *BTK* p.C481S positive subclone accompanied by expansion of the subclone harbouring *PLCG2* p.D993H which was previously dominant in the lymph node (Figure 2A). With these dynamic changes in the subclonal architecture, the disease progressed and ultimately led to the death of the patient.

Clonal evolution is a major driving force of relapse and progression of cancer, including haematological malignancies.[12] Several studies have traced the effect of treat-

ment on temporal evolutionary trajectories of CLL in the context of standard as well as targeted therapies and identified profound subclonal heterogeneity and active clonal selection in the majority of cases.[6,13,14] However, the spatial aspect of subclonal dynamics has not been appreciated until recently. Here, we presented a unique case of CLL, developing ibrutinib resistance via multiple routes simultaneously, with the observed spatial heterogeneity leading to progression and aggressive transformation of the disease. This case highlights the importance of genetic profiling of multiple affected sites as investigations restricted to peripheral blood may underestimate the repertoire of clinically relevant genetic alterations present in the patient.

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References

1. Farooqui MZ, Valdez J, Martyr S, et al. Ibrutinib for previously untreated and relapsed or refractory chronic lymphocytic leukaemia with TP53 aberrations: a phase 2, single-arm trial. Lancet Oncol. 2015;16(2):169-176.
2. O'Brien S, Furman RR, Coutre S, et al. Single-agent ibrutinib in treatment-naive and relapsed/refractory chronic lymphocytic leukemia: a 5-year experience. Blood. 2018;131(17):1910-1919.
3. Ahn IE, Underbayev C, Albitar A, et al. Clonal evolution leading to ibrutinib resistance in chronic lymphocytic leukemia. Blood. 2017;129(11):1469-1479.
4. Burger JA, Landau DA, Taylor-Weiner A, et al. Clonal evolution in patients with chronic lymphocytic leukaemia developing resistance to BTK inhibition. Nat Commun. 2016;7:11589.
5. Kadri S, Lee J, Fitzpatrick C, et al. Clonal evolution underlying leukemia progression and Richter transformation in patients with ibrutinib-relapsed CLL. Blood Advances. 2017;1(12):715-727.
6. Landau DA, Sun C, Rosebrock D, et al. The evolutionary landscape of chronic lymphocytic leukemia treated with ibrutinib targeted therapy. Nat Commun. 2017;8(1):2185.
7. Rosenquist R, Ghia P, Hadzidimitriou A, et al. Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: updated ERIC recommendations. Leukemia. 2017;31(7):1477-1481.
8. Albitar A, Ma W, DeDios I, et al. Using high-sensitivity sequencing for the detection of mutations in BTK and PLCgamma2 genes in cellular and cell-free DNA and correlation with progression in patients treated with BTK inhibitors. Oncotarget. 2017;8(11):17936-17944.
9. Agathangelidis A, Ljungstrom V, Scarfo L, et al. Highly similar genomic landscapes in monoclonal B-cell lymphocytosis and ultra-stable chronic lymphocytic leukemia with low frequency of driver mutations. Haematologica. 2018;103(5):865-873.
10. Damm F, Mylonas E, Cosson A, et al. Acquired initiating mutations in early hematopoietic cells of CLL patients. 2014;4(9):1088-1101.
11. Komarova NL, Burger JA, Wodarz D. Evolution of ibrutinib resistance in chronic lymphocytic leukemia (CLL). Proceedings of the National Academy of Sciences of the United States of America. 2014;111(38):13906-13911.
12. Landau DA, Carter SL, Getz G, Wu CJ. Clonal evolution in hematological malignancies and therapeutic implications. Leukemia. 2014;28(1):34-43.
13. Landau DA, Carter SL, Stojanov P, et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. Cell. 2013;152(4):714-726.
14. Schuh A, Becq J, Humphray S, et al. Monitoring chronic lymphocytic leukemia progression by whole genome sequencing reveals heterogeneous clonal evolution patterns. Blood. 2012;120(20):4191-4196.