Epigenomic evolution in chronic lymphocytic leukaemia cells from patients treated with ibrutinib and showing disease progression

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Running title: Epigenomic changes in ibrutinib-treated CLL cells
Abstract

Background
The Bruton’s tyrosine kinase (BTK) inhibitor ibrutinib provided a breakthrough in the treatment of chronic lymphocytic leukaemia (CLL), but cases of resistance are now emerging. Whilst resistance is commonly associated with mutations in BTK itself and the downstream signalling molecule PLCγ2, this is not always the case. In a recent study, we have detected some epigenomic plasticity correlated with the dynamics of CLL cell response to ibrutinib. To understand the mechanisms mediating resistance in CLL, it is important to be able to determine whether the observed phenotypic changes are driven solely by resistance cues (e.g. clonal evolution, activation of signalling bypassing BTK inhibition), or if they could also be the consequence of unrelated events.

Methods
To answer to this question, we have monitored chromatin changes happening in response to ibrutinib from the start of treatment until relapse in CLL cells from both a patient carrying a previously identified BTK C481S mutation and from a relapsing patient, for whom none of the classical genetic lesions associated with ibrutinib relapse was detected.

Results
We established that the epigenome and gene expression in CLL cells from patients on ibrutinib changes with time independently of disease progression and identified two patterns of chromatin alterations, which are independent of resistance mechanisms; one ibrutinib-dependent and the other related to quiescence. Furthermore, by defining the main characteristics of resistance-independent epigenomic plasticity and excluding them from the analysis, we identified candidate genes potentially mediating disease progression.

Conclusion
This two-step strategy could fundamentally alter the understanding of resistance to
treatment in CLL.

Key words: CLL, ibrutinib, relapse, epigenome, plasticity
Background

Chronic lymphocytic leukaemia (CLL), the most frequent leukaemia in western countries, originates from clonal proliferating B-cells (1, 2). Treatment with the Bruton's Tyrosine Kinase (BTK) inhibitor ibrutinib has shown extremely promising results in front-line treatment as well as in relapsed/refractory CLL (3, 4) and is now being tested in combination with other therapies (5-7). However, a significant number of cases of both primary and secondary resistance have been reported (8, 9) and when treatment is discontinued, long-term outcome is generally poor (10).

Intensive efforts are undertaken to better understand the mechanism of response to ibrutinib and the development of drug resistance. In CLL cells, the initial response is a sharp downregulation of the NF-κB and BCR signalling pathways (11-14) and a reduced activity of lineage specific transcription factors (14). This is followed by ibrutinib-dependent lymphocytosis (15), cells from lymph nodes being expelled into the peripheral blood (PB). These cells are typically over-expressing genes related to proliferation pathways and cell cycle (16). Accordingly, epigenomic analysis of these cells reveals transient increases in histone H3 lysine 4 trimethylation (H3K4me3), a histone post-translational modification (PTM) associated with open chromatin at cis-elements targeted by lineage defining transcription factors providing a signature of activation (17). Longer exposure to ibrutinib induces erosion of CLL identity correlated with a quiescent-like gene expression signature (14), global loss of both H3K27 acetylation (activating) and trimethylation (repressive) marks (17). Furthermore, while H3K4me3 stays globally relatively stable with time on ibrutinib, this histone PTM is distinctly reduced in regions identified as bivalent (H3K4me3+/H3K27me3+) before treatment (17). All of these observations indicate an important epigenomic plasticity of CLL cells in response to ibrutinib treatment.

Relapsed CLL, after ibrutinib therapy, is commonly associated with genomic alterations in BTK and its downstream target PLCγ2. However, cases of progressive disease in which targeted sequencing is unable to explain resistance have also been reported (12...
and activation of alternative signalling pathways that bypass BCR signalling might also play a role in such process.

In the present analysis, we monitored chromatin structure evolution in CLL cells from patients treated with ibrutinib before and after disease progression (Supplementary tables 1 and 2). In contrast with genetic abnormalities, the specificity of the chromatin landscape for a given individual correlates with cell phenotype and is, consequently, relevant for this particular individual when compared to clinical outcome. This study, combined with gene expression analysis, suggests coordinated phenotypic variations of CLL cells independent of disease evolution, characteristic of either cell activation or quiescence, consistent with our previous observations (17), and not explained by technical variability. We also determined that WNT5A might participate in the mechanism of relapse in a patient with no detectable mutation of either the BTK or PLCγ2 genes.

Results

Chromatin structure varies with time in CLL cells from patients on ibrutinib

We have previously analysed the evolution of the chromatin structure in CLL cells from patients enrolled in the IcICLLe trial, a feasibility study investigating the mechanism of action of ibrutinib. Patients received continuous oral therapy with ibrutinib (420 mg OD) and CLL cells were collected from PB before and at regular time points during treatment (17). From this trial, we identified a CLL patient (IbruR3) who progressed after 24 months of ibrutinib therapy (Supplementary Fig. S1A). We screened for previously identified mutations associated with relapse (18, 19). Interestingly, we detected the previously characterised BTK C481S mutation after 32 months, but not at month 30 even though the percentage of CLL cells in the PB was already notably increasing (Fig. 1A, Supplementary Fig. S1A). In addition, the level of H3K27me3 and H3K27ac was low at 6 months post treatment initiation for this patient, in agreement with our previous observation showing global disappearance of both histone marks.
after months on ibrutinib (17) and was elevated at 33 months corresponding with progression.

The 9 months’ time point showed a normal level of H3K27me3 as well as a slight and global
increase in H3K27ac compared with 3 months earlier (Fig. 1B and C, Supplementary Fig.
S1B). However, CLL cell count was decreasing at 9 months suggesting that the patient was
responding well to the treatment at that time (Supplementary Fig. S1A). Furthermore, we
compared this observation with the analysis of the chromatin structure of CLL cells obtained
from peripheral blood samples from a patient with acquired resistance to ibrutinib (IbruR1),
who was not enrolled in the trial. For this patient, we analysed chromatin changes in two
samples collected within an interval of 3 months, both after the diagnosis of relapse. PCR
screening for mutations including BTK C481S and the PLCγ2 L845F, R665W and S707Y
mutations (18, 19) was negative for both time points. A genome-wide increase in the monitored
histone marks was observed in this patient for the first time point, however, a noticeable
reduction in both H3K27me3 and H3K27ac was observed 3 months later, suggesting that
disease progression might not be a continuous process (Fig. 1B and C, Supplementary Fig.
S1B and C). Altogether, these data suggested that some chromatin changes are dependent
and some independent of disease evolution.

H3K27ac can vary independently of disease progression

To further characterise the observed changes and determine if technical variability
could be ruled out, we performed a quantitative analysis of H3K27ac evolution compared to
before treatment. Loss or reduction in H3K27ac was global and partially restored at 33 months
compared to before treatment (Fig. 2A). Interestingly, the global change of H3K27ac peak
intensity was comparable between IbruR3 before treatment and in the progressing sample
(33M) and between the 3 months interval samples for IbruR1, suggesting a similar reduced
transcriptional activity in both later time points (Fig. 2A).

Moreover, when comparing this histone mark at 9M and 6M (IbruR3), we observed
that 56% and 11% of these peaks were higher at 9M and 6M respectively (Fig. 2B,
Supplementary Fig. S1D). Gene ontology (GO) analysis of the nearest genes associated with
the selected peaks, did not reveal any specific functional association for the 6M high peaks but the 9M high fraction was enriched for genes of the canonical WNT and TGFβ-signalling pathways as well as genes associated with regulation of cell growth (Supplementary Fig. S2A Supplementary Table 4). Next, we analysed the intersection between our peak libraries and merged transcription factor ChIP-seq data obtained from a publicly available database (ReMap). This analysis, referred to as REMI, calculates the number of cis-regulatory elements enriched for a specific histone mark and containing a validated consensus sequence corresponding to a specific transcription factor (17). This allowed us to generate signatures associated with determined peak populations and to assess the relative variation of these signatures when comparing one peak population to another (17). Using REMI analysis, we identified an enrichment for peaks containing validated binding-sites for the mediator complex (CDK8, MED12) and the Polycomb Receptor Complex 2 (PRC2) (EZH2, SUZ12) in the 9M high peak population and for SOX2 and PRDM1 in the 6M high one (Fig. 2C).

The same analysis performed to compare the H3K27ac peak population between IbruR1 and IbruR3 BT suggested that histone acetylation was increased for genes of the stress activated signalling cascade or cell-cell adhesion in the nearest genes associated with peaks high in IbruR1 or IbruR3 cells respectively (Supplementary Fig. S2B, Supplementary table 4). Interestingly, EZH2 and SUZ12 functional binding sites were found in the decreased H3K27ac peaks fraction and SOX2 in peaks enhanced in IbruR1 compared to IbruR3, respectively (Fig. 2D). Moreover KLF4, which was among the genes with higher level of H3K27ac at associated cis-regulatory elements in IbruR3 compared to IbruR1, was also found enriched in the IbruR3 high peak population (Fig. 2D, Supplementary table 4). Altogether these data suggested that loss of H3K27ac at EZH2/SUZ12-associated peaks was correlated with a gain in SOX2-associated peaks in these cells, such coordinated changes ruling out technical artefacts.

Changes in H3K4me3 peak number follow H3K27ac evolution
We have shown previously that H3K4me3 was progressively disappearing at bivalent cis-elements and transiently increasing at lineage specific cis-elements (LSCE) (17). The latter changes were correlated with cells entering the peripheral blood from the proliferation centre, which could be labelled as activated-CLL cells (17).

Here, comparing H3K4me3 peak number evolution between our different time points, we observed dynamic variations concordant with those seen for H3K27ac. These variations reached a maximum of 16% peak loss observed at 6M compared to 2M for IbruR3 and 30% loss between IbruR1 and IbruR1 +3M (Fig. 3A). To further characterise these evolutions, we performed a correlation study using REMI analysis first looking at this peak evolution compared to IbruR3 before treatment (IbruR3 BT) and then versus IbruR3 2M. As observed previously, the fluctuation in peak content at 2M did not exceed 20% of what is observed before treatment, with the number of bivalent peaks (EZH2, SUZ12) going down and LSCE peaks going up and this fraction being enriched for cis-elements containing functional binding sites for SOX2 and PRDM1 (Fig. 3B). Overall, the score evolution could be separated into 4 subsets, (1) LSCE, (2) bivalent, (3) PRDM1/SOX2 and (4) the other transcription factors (TFs) of the ReMap database (Fig. 3C-I). In all samples analysed including IbruR3 2M, bivalent peaks were down and SOX2/PRDM1 high suggesting that it could be a characteristic of ibrutinib-treated CLL cells (Fig. 3C-I). Furthermore, we identified several types of CLL cell populations based on the evolution of both LSCE and the rest of the TFs. First, as expected for the three subsets showing a reduction of the total H3K4me3 peak number, 6M, 9M, and IbruR1 +3M (Fig. 3A), an increased proportion of all the other TF-associated peaks (subset 4) was observed, suggesting a loss of non-promoter cis elements (small) and a proportional enrichment for promoters (larger cis-elements containing more TF-binding sites) (Fig. 3C, D and G). In addition, the number of H3K4me3 LSCE-associated peaks could be separated into (1) high, between 10% and 30% increase for IbruR1 and (2) low, less than 20% increase for the other samples (Fig. 3C, D and G). Furthermore, IbruR1 peak score was highly correlated with a sample previously characterised as activated CLL-cells (TN_7d, R² = 0.95). These cells came from a front-line donor after 7 days on ibrutinib and characterised by a sharp increase
in CLL cell count in the PB in the first days of treatment (17) (Fig. 3I). Therefore, by merging these two observations combined with the global level of H3K27me3, we could separate these samples into four distinct groups; newly generated activated CLL cells (IbruR1, TN_7d), early (2M and 33M, the correlation between both peak populations being \( R^2 = 0.67 \)), intermediate (9M) and late/oldest (6M, IbruR1 +3M) quiescent-like cells (Fig. 3C-I).

The difference in H3K4me3 peak intensity is indicative of variations in transcriptional activity in these cells

The completed analyses described above suggested that CLL cells might have different phenotypes, from activated CLL cells and progressing toward different degrees of quiescence with a progressive disappearance of H3K27ac genome-wide and H3K4me3 at LSCE-associated peaks, the oldest cells also losing H3K27me3.

First, to confirm that our classification of the different subcategories of cis-elements containing identified TFs was correct, we took advantage of a new study which classified each open chromatin region in CLL into 12 chromatin states depending of their content in specific histone marks (20). Each H3K4me3 peak identified in our analysis was marked in red if they were present in a specific chromatin state and in white if not. Finally, we ranked these peaks for each analysed sample based on fold change between this sample and IbruR3 BT (Fig. 4A-G). The same analysis was performed with 60 selected TFs from the ReMap database (Supplementary Fig. S3A). By comparing both analyses, we confirmed that EZH2 and SUZ12 were enriched in the decreased fraction of IbruR1, which were enriched for the chromatin states E5, E6, E7 representing polycomb-dependent silenced and bivalent cis-elements. Similarly, H3K4me3 peaks associated with lineage specific transcription factors including SOX2, PRDM1, BCL6 and BATF were enriched in the increased fraction, which contain E1, E8 and E9 non-promoter cis-elements (Fig. 4, Supplementary Fig. S3A). Moreover, the number of peaks in this increased fraction for each CLL sample was correlated with the level of activation of these cells (Figs. 3 and 4).
To fine tune the analysis of the changes of peak population in these different samples, we separated these H3K4me3 peaks in 4 subsets depending on fold change versus IbruR3 BT and performed the same analysis as described in Fig. 3 (Supplementary Fig. S3B). Overall, the peak content for the different subsets was highly correlated between all samples with few noticeable variations (Supplementary Fig. S3C-F). As expected, bivalent regions (SUZ12, EZH2) were mainly found in the decreased subset, with IbruR1 +3M being the only sample showing a reduced number of these elements compensated by an accumulation of SUZ12-associated cis-element in the increased subset (Supplementary Fig. S3C and S3E). Moreover, further loss of bivalent cis-elements with time on ibrutinib and the reciprocal gain of SOX2-associated regions were observable for the stable and increased subsets when comparing samples collected after 6 months treatment and IbruR3 2M (Supplementary Fig. S3C and D). The most important variations could be seen when analysing the peak content of the increased subset, probably due to the small number of peaks in it (Supplementary Fig. S3C). Notably, PRDM1-containing validated binding sites were enriched at 6M, 9M and for IbruR1 and 6M, 9M, 33M and IbruR1 +3M were associated with a decrease in TAp73A-containing cis-elements (Supplementary Fig. S3C). In contrast at 33M, the increased subset was enriched for cis-elements containing validated ONECUT1- and MEIS1-binding sites and depleted from BCL6 sites suggesting a possible change in the TF network regulating proliferation after disease progression for this patient (Supplementary Fig. S3C). Finally, 6M and IbruR1 were the two samples showing no correlation when compared to IbruR3 2M in the absent subset, with the observed peak scores being indicative of an important loss of H3K4me3 at promoters (large regions targeted by multiple TFs) (Supplementary Fig. S3F). At 6M, it could be explained by the loss of almost 3000 peaks compared to 33M. In contrast, further analysis revealed that, for IbruR1, it was dependent upon promoter reorganisation and nucleosome loss as seen for selected samples (Supplementary Fig. S4A). Therefore, activated-CLL cells were characterised by an accumulation of H3K4me3 at non-promoter elements and by chromatin reorganisation leading to nucleosome loss at active promoters.
To conclude this analysis of H3K4me3 quantitative changes, we also compared the evolution of the intensity of these peaks with time to determine if these changes were random or conserved between samples (Fig. 5A). We determined that 57% of the enriched peaks seen at 9M are also enriched at 6M and 33M. Similarly, 81% of the decreased peaks at 33M are also decreased at 6M and 9M suggesting a significant level of stability between untreated and on ibrutinib treated samples (Fig. 5A). Moreover, 98% of the peaks higher in IbruR1 +3M than in IbruR3 BT were also higher three months before (Fig. 5A). We have associated those cis-elements conserved in 6M, 9M and 33M with their closest genes and determined functional associations between these genes by gene ontology analysis (Supplementary Fig. S4A, S5A, B and table 4). However, the meaning of these changes is unclear because it occurred in the context of a global decrease in H3K27ac and therefore reduced transcriptional activity. Similarly, focusing on the 98% of peak high in both IbruR1 samples, non-canonical, positive regulation of differentiation and actin cytoskeleton organisation were among the function enriched for IbruR1 compared to IbruR3 BT (Supplementary Fig. S4A, Supplementary table 4). Interestingly, genes associated with actin cytoskeleton organisation (PROX1, ACTN1, ATR, COBLL1) were found to be enriched before treatment compared to 6M-33M and reduced compared to IbruR1 (Supplementary Fig. S4B). This revealed alternative H3K4me3 peaks in regions corresponding to potential alternative promoters for IbruR1 suggesting a possible aberrant regulation of these genes in CLL cells for this first relapse patient (Supplementary Fig. S5B). To conclude, gene ontology analysis comparing genes closest to peaks enriched in IbruR1 +3M compared to IbruR3 at 6M, 9M and 33M identified several pathways specifically activated in quiescent-like IbruR1 including JNK, non-canonical WNT and pro-inflammatory signalling pathways (Supplementary Fig. S5B). JNK signalling pathway has been shown to mediate apoptosis in response to various treatment in CLL (21-23), but is inactivated by NF-κB (24).

Activation of the non-canonical Wnt signalling pathway might participate in disease progression
To identify chromatin structure specifically associated with IbruR1 and considering the fact that this first CLL sample was showing characteristics of activated-CLL cells, we compared H3K4me3 for this patient with 5 previously analysed patients for whom we had chromatin at a time point corresponding to ibrutinib-dependent lymphocytosis (17). We performed gene ontology analysis for the closest genes associated with H3K4me3 peaks found 2.5-fold higher for IbruR1 compared to all 5 other samples. Only 15 genes were found associated with specific functions, including regulation of growth and the non-canonical Wnt signalling pathways (Fig. 5B and C). Of particular interest among these genes was \textit{WNT5A}, a gene previously associated with ibrutinib resistance (25), and the fibroblast growth factor 20 (\textit{FGF20}), a ligand also associated with proliferation and developmental growth (Fig. 5C).

H3K4me3 data confirmed that this mark was specifically high at \textit{WNT5A} promoter for both IbruR1 samples and that a cis-element upstream of the \textit{FGF20} promoter was also specifically enriched for this histone mark in IbruR1, but lower at +3M (Fig. 5D).

To determine if the changes observed in chromatin structure were supported by similar changes in gene expression, we extracted mRNA from IbruR1, IbruR1 +3M, IbruR3 BT and IbruR3 33M as well as from an additional relapse patient IbruR2 and from 14 patients on ibrutinib for which we collected CLL cells from before treatment and up to 18 months post treatment initiation (Fig. 6A, Supplementary Fig S7 and S8). None of these later patients showed any sign of disease progression at the time of the study. We compared gene expression for two proliferation markers (\textit{Ki67}, \textit{PCNA}), \textit{FGF20} and \textit{WNT5A}. Among the samples from relapse patients, only IbruR1 expressed both proliferation markers reinforcing the view that these CLL cells were characteristic of activated cells and that IbruR2, IbruR3 33M and IbruR1 +3M were in a more resting/dormant stage (Fig. 6A).

Unexpectedly, \textit{PCNA} and \textit{Ki67} were also expressed in patients 7, 8, 9, 13 and 14 at various time points (Fig. 6A). A more extensive gene expression analysis indicated a close correlation between expression of these proliferation markers and genes including \textit{BCL2}, \textit{EZH1/2}, \textit{SOX11}, \textit{CD38}, a surface protein for which enzymatic activity has been correlated with enhanced growth and cellular mobility and poor risk in CLL (26), interferon and chemokine...
receptors (Supplementary Fig. S7 and S8). Similarly, expression of pro-inflammatory related
genes including \textit{TLR4}, \textit{TLR9} and \textit{TNFA} was also closely correlated, with the expression of
these genes overlapping partially with the proliferative-associated ones as seen when
comparing \textit{TNFA} expression with \textit{SOX11} or \textit{PCNA} (Supplementary Fig. S7 and S8, Fig. 6A).
Finally, the gene expression profile of IbruR1 +3M was unique showing specific expression of
\textit{WNT5A} and \textit{FGFR1} (Fig. 6A, Supplementary Fig. S8A, right part) and substantial variations
compared to 3 months earlier (Supplementary Fig. S9). \textit{FGFR1} expression was observed for
IbruR1 +3M and its ligand \textit{FGF20} was expressed in IbruR1 with a gene expression profile
closely correlated to \textit{VDR}, \textit{RXRA}, \textit{ELK1} and \textit{ILB1} (Supplementary Fig. S8A). These data
indicated that expression of two potential drivers of resistance were only detected in CLL cells
after a prolonged phase of quiescence (H3K27me3 low).

Discussion

CLL cells present significant phenotypic variations depending on their age and
compartment that they come from (27). In response to ibrutinib, after an initial phase of
lymphocytosis, the chromatin structure of CLL cells changes progressively from activated cells
toward a quiescent-like phenotype losing both H3K27ac and H3K27me3 genome-wide as well
as H3K4me3 primarily at bivalent cis-elements characteristic of resting CLL cells (14, 17).
Here, we have shown an expected chromatin and gene expression profile of activated CLL
cells in one patient on ibrutinib showing disease progression, whereas two other “relapse”
samples have chromatin characteristics of quiescent-like cells. These data suggest that
relapse is not a continuous process and as observed in the absence of treatment, that these
cells could be either in a proliferating or a quiescent phase (Fig. 6B).

Interestingly, CLL cells from patients on ibrutinib display some similar chromatin
characteristics, independent of disease progression. These include loss of H3K4me3 at
bivalent cis-elements and, reciprocally, gain of the same histone PTM at regions containing
validated SOX2-binding motifs. SOX2 is of interest as a key regulator of both the early
development and maintenance of undifferentiated embryonic stem cells and has been characterised as an oncogene in multiple cancers (28). EZH2-, SUZ12- and SOX2-validating binding sites included into the ReMap database come from studies performed in Embryonic Stem Cells (ESCs), including studies by the ENCODE consortium (29-32). Our results suggest that in response to ibrutinib CLL cells switch on or off cis-elements which are activated or repressed in ESCs respectively, linking a quiescence-like phenotype with increased ‘stemness’ as described in disseminating tumour cells (33, 34).

Furthermore, the maintenance of this signature after relapse suggests that disease progression, at least in its initial phase, occurs while cells are still in some degree sensitive to ibrutinib. For the C481S BTK mutant (IbruR3), these data agree with the fact that more than 50% of the cells do not carry the mutation in the initial phase of disease progression. This discrepancy between disease progression and the percentage of BTK mutants in the growing cell population is characteristic of ibrutinib treatment in CLL (12).

Moreover, our data suggest additional chromatin structure variations in quiescent-like CLL cells, possibly dependent on two parameters - (i) disease progression and (ii) age of the cells. EZH2 expression is restricted to proliferative cells in cancer and in non-transformed human cells (35) and, accordingly, to proliferation centres in CLL and to the germinal centre in B cells (36-38). Consequently, loss of H3K27me3 in quiescent-like CLL cells can be interpreted as a prolonged exit of these cells from the cell cycle, characteristic of aging cells. Here, two CLL samples amongst those investigated at greatest depth, IbruR3 6M and IbruR1 +3M, share a profile of drastic reduction in H3K27me3 suggesting that both have been maintained in G0 phase for an extended period of time. Interestingly, these samples also show common characteristics associated with quiescent-like cells including loss and gain of H3K4me3 positive cis-elements associated with Tap73 and PRDM1, respectively. Tap73 promotes the Warburg effect and proliferation in cancer (39); therefore, silencing of Tap73-associated cis-elements might be a characteristic of this quiescence. Similarly, PRDM1/Blimp1, a master regulator of B cell differentiation, has been shown to actively repress a number of
functions in B cells including cell-cell adhesion (40), which we show to be associated with an increase in H3K4me3 at 6M, 9M and 33M compared to pre-treatment.

Whilst the IbruR1 +3M epigenome is comparable to that of IbruR3 6M, suggesting a prolonged exit from the cell cycle, the level of H3K27ac is significantly higher in these cells, indicative of enhanced transcriptional activity matched by a gene expression profile which is distinct from other quiescent-like cells. Additionally, these cells show an enrichment for H3K4me3 at bivalent regions, which could be due to the concomitant loss of the repressive mark (H3K27me3) and an increase in global transcriptional activity. In these cells, \textit{WNT5A} and \textit{FGFR1}, which have been described as potential candidates to mediate ibrutinib-resistance, show 44- and 17-fold higher expression compared to cells three months earlier, which have an activated phenotype. \textit{WNT5A} can enhance the proliferation and migration of CLL cells via activation of the non-canonical WNT pathway (25, 41), a mechanism which is not inhibited by ibrutinib (42). Similarly, activation of FGFR targets four internal signalling pathways RAS-MAPK, PI3K-AKT, PLC\textsubscript{γ}, and STAT (43), suggesting that it might also induce proliferation in CLL cells by bypassing BTK inhibition. These pathways may act synergistically, with canonical WNT5A signalling increasing the transcription of \textit{FGF20}, an FGFR1 ligand (44).

Detecting transient expression of proliferation markers, including \textit{Ki67} and \textit{EZH2}, in cells from four patients showing no clinical sign of disease progression reinforced the view that CLL cells are still able to enter cell cycle under ibrutinib-dependent repression of the BCR signalling pathway, as shown by analysis of IbruR3 9M epigenome. These data argue that CLL cells from patients treated with ibrutinib are still able to enter cell cycle in the absence of detectable disease progression.

\textbf{Conclusions}

We have shown that the chromatin structure of CLL cells from patients on ibrutinib changes dynamically for reasons independent of sensitivity to the treatment. These changes are coordinated and consistently correlated with the age of the cells and cannot be explained by random variations due to technical inconsistency. Accordingly, CLL cells from relapse
patients can be either in a quiescent-like or proliferating state. In addition, our data suggest that older cells are the ones which might be responding to signalling which is capable of bypassing BTK inhibition. As mentioned in the introduction, these phenotype-associated variations are relevant for the particular individuals included in this study and suggest that any mechanistic analysis aimed at identifying the phenotypic specificity of relapsing cells would have to take the inherent epigenomic variation into consideration.

**Methods**

**Isolation of CLL Cells**

Peripheral blood was collected from patients with informed written consent (St. James’s University Hospital, approval number 14/YH/0034; IclICLLe EudraCT number: 2012-003608-11). Peripheral blood mononuclear cells (PMBCs) were isolated by density gradient centrifugation using Lymphoprep™ (Axis-Shield). CLL cells were further purified using CD19+ MicroBeads (Miltenyi Biotec).

**BTK and PLCγ2 Mutation Detection**

100 ng of patient DNA extracted from CD19+ selected peripheral blood was used for PCR. A 527 bp fragment encompassing the BTKCys481 region was amplified using the following primers, GACTGCTCTGATTCCCACCA and TACTCCTAGGTCAGCCCCTTC. Similarly, three regions of the PLCγ2 gene were amplified, a 190 bp fragment which encompassed the PLCγ2S707 region using the primers ACCAGGATCTTGGCATGTCA and GGGTAGCGCAGTCTCATCTT, a 323 bp fragment encompassing the PLCγ2L845 region using the primers, TCCCCATGGAGCCTTGGTGCTAGCCG and a 212 bp fragment covering the PLCγ2R665 region using the primers AGGTGTCACTGGTCCATTGA and AGGTGATGGCATAGGAGTC. The amplified products were isolated using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and then sent for Sanger sequencing (Source Bioscience, Nottingham, UK).
Immunoblot Analysis

Immunoblot analysis was conducted as published previously (17). Primary antibodies were as follows: H3K4me3 (Millipore; 04-745), H3K27me3 (Millipore; 07-449) and H3K27ac (Millipore; 07-360) were used as primary antibodies and anti-Total H3 (Abcam; ab1791) was used as loading controls.

Chromatin Immunoprecipitation (ChIP) and ChIP-seq

ChIP was performed as previously described (45, 46) using Dynabeads protein G (Invitrogen) with 2.4 mg per 10 ml beads with 1 μg anti-H3K4me3 (Millipore; 04-745), anti-H3K27ac (Millipore; 07-360), anti-H3K27me3 (Millipore; 07-449). For sequencing (ChIP-seq), libraries were prepared using NEBNext Ultra™ II DNA Library Prep Kit (New England Biolabs). See Supplementary Methods for sequencing run details.

Bioinformatics Analysis

See Supplementary Methods for further details. Sequence data from this study have been submitted to ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-8220.

Gene Expression Analysis

Total RNA was extracted from patient CLL samples using Direct-zol™ RNA MiniPrep Kit (Zymo Research) and cDNA was prepared using M-MLV Reverse Transcriptase (Invitrogen). Expression of target genes (Supplementary Table 2) was determined using quantitative real-time polymerase chain reaction (qPCR) with GoTaq® qPCR Master Mix (Promega) on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). Relative expression (ΔCt) was calculated from the Ct value of the average of 5 housekeeping genes (PPP6C, GAPDH, RPL13A, β-Actin, B2M) – Ct value of the gene of interest.
list of abbreviations

ACTN1 actinin alpha 1
BATF basic leucine zipper ATF-like transcription factor
BT before treatment
BTK bruton’s tyrosine kinase
B2M beta 2 microglobulin
CDK8 cyclin dependent kinase 8
CLL chronic lymphocytic leukaemia
COBLL1 cordon-bleu WH2 repeat protein like 1
ESC embryonic stem cell
EZH2 enhancer of zeste 2 polycomb repressive complex 2
FGF20 fibroblast growth factor 20
FGFR1 fibroblast growth factor receptor 1
ILB1 interleukin 1 beta
GAPDH glyceraldehyde 3 phosphate dehydrogenase
GO gene ontology
IbruR ibrutinib resistant
JNK c-Jun N-terminal kinase
Ki67 marker of proliferation Ki-67
KLF4 kruppel like factor 4
LSCE lineage specific cis-elements
MAPK map kinase
MED12 mediator complex subunit 12
NF-κB nuclear factor kappa B
ONECUT1 one cut homeobox 1
PB peripheral blood
PCNA proliferating cell nuclear antigen
PI3K phosphatidylinositol 3 kinase
PLCγ2 phospholipase C gamma 2
PMBC peripheral blood mononuclear cell
PPP6C protein phosphatase 6 catalytic subunit
PRC2 polycomb receptor complex 2
PRDM1 PR/SET domain 1
PROX1 prospero homeobox 1
PTM post-translational modification
qPCR quantitative real-time polymerase chain reaction
RPL13A ribosomal protein L13a
REMI reMap and epigenetic mark intersection
RXRA retinoid X receptor alpha
SOX2 SRY-box transcription factor 2
SOX11 SRY-Box transcription factor 11
STAT signal transducer and activator of transcription
Tap73 transformation related protein 73
TGFβ transforming growth factor beta
TF transcription factor
TLR Ttoll like receptor
TN treatment naive
TNFA tumor necrosis factor
VDR vitamin D receptor
WNT5A wnt family member 5A

Declarations
Ethical approval and consent to participate

Peripheral blood was collected from patients with informed written consent (St. James's University Hospital, approval number 14/YH/0034; IclCLLe EudraCT number: 2012-003608-11).

Consent for publication

Not applicable

Availability of supporting data

Sequence data from this study have been submitted to ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-8220.

Competing interests

The authors declare no competing financial interests.

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Authors’ contribution

KH, FT, CE and AR performed the experiments and generated figures. PL, IS analysed the data and PL wrote the paper. PH was involved in the initial design of the project and contributed to provide essential research material. DN participated to the data analysis. KH, IS, PH and DN participated to the redaction of the manuscript.

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**Figure legend**
Fig. 1: Overview of chromatin structure changes with time on ibrutinib. (A) Identification of the BTK C481S mutation by PCR after 32 months of ibrutinib treatment. The black arrows indicate the position of the cysteine codon (UGC) mutated into serine (UCC). (B) Summary of the ChIP-seq data focusing on two regions (chr18:27mb-37mb and Chr11:91,000-98,000) representative of the obtained data and including a gene rich region surrounded by two gene poor regions for which H3K27m3 enrichment is at background level. ChIP-seq has been performed for H3K27me3, H3K4me3 and H3K27ac looking at two patients (1) IbruR3 after 6, 9 and 33 months treatment and (2) IbruR1 at the time of disease progression detection (a) and 3 months later (+3M).

Fig. 2: Global level of H3K27ac does not follow disease progression. (A) Differential H3K27ac, as presented in Fig. 1 and defined as log₂(H3K27ac fold-change) and log₁₀(-p-value) between IbruR3 before treatment and at 6, 9 and 33 months as well as between IbruR1 and IbruR1 +3 months. Each H3K27ac peak was associated with its closest gene. (B) Same as A, but comparing IbruR3 at 6 and 9 months. (C) REMI analysis, cis-elements enriched for the H3K27ac mark were screened for the presence of validated binding sites for transcription factors and co-regulators (ReMap database). For each protein of the database, data were expressed as a score (equation (2) in Supplementary methods) corresponding to the number of peaks associated with a specific TF at 9M versus 6M. (D) Same as D but comparing IbruR3 BT and IbruR1.

Fig. 3: IbruR1 is presenting a H3K4me3 profile of activated-CLL cells. (A) Total H3K4me3 peak number between the different analysed subsets including IbruR3 BT, IbruR3 2M and TN 7d from a previous study (17). (B) REMI analysis, score variation for IbruR3 2M compared to before treatment. (C-I) Scatter diagrams representing a comparison between REMI scores for the different analysed samples (y axes) and reference profile (C-E, G and H) IbruR3 2M, (F) IbruR3 6M and (I) TN 7d (x axes).
Fig. 4: Changes in chromatin structure depending of the chromatin state. (A-G) Bar coding presentation: chromatin states as defined previously (20) (E1 and E9, H3K4me1/H3K27ac; E8, H3K4me1; E10, H3K4me3; E11, H3K27ac/H3K9ac; E5 and E6, H3K27me3; E7, H3K27me3/H3K4me1/3) have been attributed a colour code depending of their overlap with identified H3K4me3 peaks in lbruR1 and/or lbruR3 samples (red = present, white = absent). H3K4me3 peaks have been ranked based on fold-change between (A) TN 7d, (B) lbruR3 2M, (C) lbruR3 6M, (D) lbruR3 9M, (E) lbruR3 33M, (F) lbruR1 and (G) lbruR1 +3M compared to lbruR3 BT.

Fig. 5: H3K4me3 peak intensity variation showed a significant degree of time dependent conservation. (A) Differential H3K4me3, as presented in Fig. 1 and defined as log$_2$(H3K4me3 fold-change) and log$_{10}$(-p-value) between lbruR3 before treatment and at 6, 9, 33 months and lbruR1 as well as between lbruR1 and lbruR1 +3 months. Each H3K4me3 peak was associated with its closest gene. The percentage of peaks conserved at all time points 6M, 9M and 33M are indicated for the decreased (red rectangle) and increased (green rectangle) fractions. Similarly, the percentage of peaks conserved in both lbruR1 and lbruR1 +3M are indicated for the increased (grey rectangle) fractions. (B-C) Biological processes enriched at genes associated with differentially regulated H3K4me3 peaks (lbruR1 compared to 5 CLL samples from patients which received up to 7 days ibrutinib treatment (17); hypergeometric test BH-FDR < 0.05). (D) Example of differential H3K4me3 peak intensity between the analysed samples (WNT5A and FGF20).

Fig. 6: WNT5A is specifically expressed in lbruR1. (A) gene expression analysis of CLL cells from PB for 14 patients collected before treatment or at 6, 9, 12 and 18M (as indicated on the Figure) compared to lbruR1 (R1), lbruR2 (R2) and lbruR (R3). PCNA, Ki67, FGF20 and WNT5A. Data are expressed relative to an average of 5 different control genes. (B) Simplified model highlighting different level of transcriptional activity in CLL cells on ibrutinib.
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Supplementary Files

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- Supplementaryfigures.pdf