In Human Visualization of Ibrutinib-Induced CLL Compartment Shift

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AbSTRACT

Bruton tyrosine kinase inhibitor ibrutinib is effective in treating chronic lymphocytic leukemia (CLL). However, after ibrutinib treatment initiation, patients frequently experience an increase of CLL blood cell count. This phenomenon in clinical practice is thought to reflect a “compartment shift” of CLL cells from lymph nodes to the peripheral blood, but the actual shifting has not yet been demonstrated. Using [68Ga]Pentixafor-PET/MRI for in vivo CXCR4 visualization, we here provide images of topical changes of CLL cells upon ibrutinib treatment. Within the first month of ibrutinib treatment, mean standardized [68Ga]Pentixafor uptake decreased in the bone marrow and lymph nodes, whereas [68Ga]Pentixafor uptake increased in the spleen. Leukocytosis rose, as did numbers of CXCR4high (tissue-resident) CLL cells. Volumes of lymph nodes and spleen decreased. Upon longer ibrutinib treatment, leukocytosis decreased, followed by a decrease of [68Ga]Pentixafor uptake in the spleen. These results support the preexisting clinical hypothesis of a “compartment shift” of CLL cells from the lymph nodes to the peripheral blood, but also refine the mechanistic model by describing early clearing of the bone marrow and redistribution of CLL cells to the orthotopic splenic cavernous system in response to ibrutinib treatment.

Introduction

Chronic lymphocytic leukemia (CLL), the most common leukemia in Western countries, involves blood, bone marrow, and lymphoid tissues, and is usually incurable (1). B-cell receptor signaling drives this disease. Targeting B-cell signaling through irreversible inhibition of Bruton tyrosine kinase (BTK) by ibrutinib has improved the prognosis of patients with CLL (2–4). Ibrutinib treatment has become the standard of care and has advanced to the first-line setting (3, 4). After initiation of treatment with ibrutinib, patients frequently experience an increase of CLL cells in the blood, a response seen with other BTK inhibitors (5, 6). The hypothesized model of this phenomenon is a “compartment shift” of CLL cells from lymph nodes to peripheral blood. The actual shifting, however, has not been demonstrated because CLL cell tracking has not been possible.

CT, an imaging technique, is used to assess response of CLL in clinical trials, as recommended by the International Workshop on Chronic Lymphocytic Leukemia (twCLL; ref. 7). Treatment response is captured as size reduction of lymphadenopathy and splenomegaly.

Changes related to the redistribution of CLL cells from tissues to the peripheral blood, however, are not detected using CT or other established imaging techniques.

High expression of CXCR4 on CLL cells is associated with a poor prognosis (8). Blocking of BTK might affect adhesion molecules and chemokine receptors, including CXCR4 and CXCR5 (9, 10), thus interfering with the tissue microenvironment and mobilizing tissue-resident CLL cells from lymph nodes into the peripheral blood (5, 11, 12). Positron emission tomography (PET) with [68Ga]Pentixafor, a radiotracer that specifically targets the CXCR4 receptor (13, 14), forms the basis of direct and sensitive detection of CLL in vivo (15). Here we use [68Ga]Pentixafor-PET/MRI to assess CXCR4 density. We indirectly assess cell density by means of diffusion-weighted MRI (DWI). We also use MRI to assess tumor volumes and spleen diameters. From these data, we provide a view of comprehensive topical changes of CLL cells upon ibrutinib treatment.

Methods

Patients

[68Ga]Pentixafor-PET/MRI was performed in 9 consenting patients with CLL before and after treatment with ibrutinib was initiated (continuous oral administration of 420 mg of ibrutinib). Short-term [68Ga]Pentixafor-PET/MRI follow-up (<1 month) was available in 4 patients: 1 week (patient 1), 2 weeks (patients 2 and 4), and 3 weeks (patient 3) after treatment start. Mid-term PET/MRI follow-up was available in 3 patients: 11 weeks (patients 5 and 6) and 18 weeks (patient 7) after treatment start. Long-term follow-up was available in 3 patients: 34 weeks (patient 8), 52 weeks (patient 1, second follow-up), and 150 weeks (patient 9) after start of ibrutinib. Time points for short-term follow-up were chosen because the peak of ibrutinib-induced leukocytosis is expected within the first 28 days after treatment initiation (16). Detailed patient characteristics are provided in Supplementary Table S1. The case series was approved by the Ethics Committee of the Medical University of Vienna (Vienna, Austria; protocol number 1655/2016), and each patient provided written,
informed consent, in accordance with institutional guidelines and the Declaration of Helsinki.

PET/MRI

Whole-body [68Ga]Pentixafor-PET/MRI before and during treatment with ibrutinib was performed on a hybrid PET/MRI device (Siemens Biograph mMR) 60 minutes after the intravenous administration of 150 MBq of [68Ga]Pentixafor (17), with 5 minutes per bed position, four iterations, 21 subsets, a 4.2-mm slice thickness, and a 172 × 172 matrix for PET. MRI was performed using a previously described protocol including axial T1-weighted and coronal T2-weighted MRI, as well as DWI (15). Maximum and mean standardized [68Ga]Pentixafor uptake values (SUV\text{max}, SUV\text{mean}) as well as total PET-based tumor volumes (PTV, in cm\(^3\)) were independently measured for involved lymph nodes, the bone marrow (including the entire spine and pelvis, as well as the sternum and ribs), and the spleen, based on isocountour volumes of interest (VOI) that were constructed using the Beth Israel PET/CT viewer plugin for FIJI (http://fiji.sc). Total morphologic tumor volumes (VOL, in cm\(^3\)) of the involved lymph nodes and the spleen were measured on T1-weighted MRI by manual delineation. The vertical diameter of the spleen was measured on T2-weighted MRI. Finally, DWI-based mean apparent diffusion coefficients (ADC\text{mean}, in \(\mu\)m\(^2\)/second), which indirectly capture cell density through the assessment of tissue diffusivity (18), were measured for the lymph nodes and the spleen.

Flow cytometry

For patients 1 to 3, Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB) isolated peripheral blood mononuclear cells (PBMC) were stained with fluorescence-labeled antibodies from BioLegend according to the manufacturer’s recommendations. The panel consisted of antibodies against CD19 (APC-conjugated, clone: HIB19), CD5 (APC-Cy7-conjugated, clone: L17F12), and CXCR4 (BV605-conjugated, clone: 12G5). Measurements were performed on a BD FACSAria Fusion flow cytometer (BD Biosciences). The gating strategy is shown in Supplementary Fig. S1.

Single-cell RNA sequencing

Single-cell RNA sequencing (scRNA-seq) data is publicly available as a Gene Expression Omnibus (GEO) dataset (GSE109085) provided by Gaiti and colleagues (19). This dataset comprises single-cell transcriptomic data from 3 patients before and 1 month after initiation of ibrutinib treatment, when ibrutinib-induced leukocytosis was detected in each patient. Data from 96 single CLL cells are available for each time point. Single cells were obtained by FACS for DAPI\(^-\)CD19\(^+\) cells in this study.

Statistical analysis

Data were analyzed using the Seurat package (20) developed for the R project statistical software suite (version 3.6.0; URL: https://www.R-project.org).

Results

Short-term [68Ga]Pentixafor-PET/MRI follow-up

Upon short-term (less than 1 month) ibrutinib treatment, SUV\text{mean} decreased in the bone marrow (1 week: −9.8%; 2 weeks: −14.7%; 3 weeks: −15.8%) and lymph nodes (1 week: −12.0%; 2 weeks: −27.3%; 3 weeks: −41.9%) of patients 1 to 3; spleen PTV (1 week: −21.2%; 2 weeks: −32.8%; 3 weeks: −18.4%) and lymph node PTV (1 week: −32.5%; 2 weeks: −42.2%; 3 weeks: −33.1%) also decreased (Fig. 1; Supplementary Fig. S2; Supplementary Table S2). In contrast, SUV\text{mean} increased in the spleen in short-term scans (1 week: +29.8%; 2 weeks: +133.0%; 3 weeks: +26.1%; Fig. 1; Supplementary Fig. S2) along with an increased leukocytosis (1 week: +65.2%; 2 weeks: +381.6%; 3 weeks: +75.1% white blood count (WBC)) and a rise of CXCR\text{high} (tissue-resident) CLL cells (1 week: +56.7%; 2 weeks: +39.1%; 3 weeks: +58.3%; Figs. 1 and 2; Supplementary Table S2). As a negative control, we also scanned a patient with low-volume lymph node involvement who did not develop increased leukocytosis upon ibrutinib initiation (−2.1% WBC after 2 weeks on ibrutinib). In line with our hypothesis, this patient did not demonstrate increased SUV\text{mean} in the spleen (−2.3%); bone marrow and lymph node SUV\text{mean} and splenic PTV showed only a small decrease (−14.4%, −11.6%, and −14.5%, respectively), whereas lymph node PTV markedly decreased (−66.1%; Supplementary Fig. S3; Supplementary Table S2).

Volumes of lymph nodes of short-term–treated patients decreased (−21.1%, −21.1%, −48.0%, and −66.1%) during ibrutinib treatment, as did spleen volumes (−23.4%, −23.5%, −20.3%, and −8.6%, respectively (Fig. 1; Supplementary Figs. S2 and S3; Supplementary Table S2). In patients 1 to 3, the respective ADC\text{mean} increased in the lymph nodes (1 week: +53.8%; 2 weeks: +53.0%; 3 weeks: +15.3%) as a sign of cell density reduction, but the opposite effect was observed in the spleen, which demonstrated a moderate ADC\text{mean} decrease (1 week: −8.1%; 2 weeks: −15.1%; 3 weeks: −11.1%) on ibrutinib (Supplementary Table S2) indicating increased cell density. In the negative control patient (who had low-volume lymph node involvement), ADC\text{mean} in the lymph nodes and the spleen remained unchanged (−1.0% and +1.6%).

Characterization of CLL cells of patients 1 to 3 by flow cytometry revealed an increase of CXCR\text{high} cells in the peripheral blood after initiation of ibrutinib therapy (1 week: 56.7% increase relative to baseline; 2 weeks: 39.1% increase relative to baseline; 3 weeks: 58.3% increase relative to baseline; Fig. 2). In addition, CXCR4 expression changes were confirmed in an independent, publicly available scRNA-seq dataset (GSE109085; made available by Gaiti and colleagues; ref. 19). Single-cell data from a total of 576 CLL cells was available for analysis (96 cells sequenced for 3 patients before and after initiation of ibrutinib therapy). Although principal component analysis (PCA) revealed distinct clusters for each patient regardless of therapy status, average expression of CXCR4 increased in all 3 patients during ibrutinib therapy (Fig. 3A). The increase of CXCR4 expression relative to baseline was 55.1% (CLL_03), 29.9% (CLL_04), and 22.0% (CLL_05; Fig. 3B). In this study, on-treatment samples were collected 1 month after initiation of ibrutinib therapy, when ibrutinib-induced leukocytosis was detectable, which is comparable with our patient cohort with short-term (less than 1 month) ibrutinib treatment (19).

Mid- and long-term [68Ga]Pentixafor-PET/MRI follow-up

After 2 to 4 months of ibrutinib treatment, patients demonstrated a decreased bone marrow SUV\text{mean} (−17.6%, −22.5%, and −16.4%) along with decreased lymph node SUV\text{mean} (−1.3%, −35.4%, −32.5%), a still increased splenic SUV\text{mean} (+34.2%, +59.6%, and +23.2%), and moderate or resolved leukocytosis (Fig. 1B; Supplementary Table S3). Similar to short-term (less than 1 month)–treated patients, PTVs and volumes of lymph nodes and spleen also decreased, and lymph node ADC\text{mean} increased, whereas ADC\text{mean} in the spleen again increased, consistent with increased cell density (Supplementary Table S3).
Long-term (more than 6 months) follow-up after ibrutinib treatment initiation demonstrated decreased leukocytosis, along with decreased bone marrow SUVmean, lymph node SUVmean, and volume, and splenic SUVmean and volume, relative to baseline (Fig. 1B; Supplementary Table S3). In patient 1, ADCmean in the spleen increased relative to the short-term follow-up (+4.2%), indicating further reduced cell density; lymph node and splenic PTVs decreased again (−80.8% and −52.7%; Supplementary Table S2).

Discussion

In patients with leukocytosis at short-term follow-up (defined as 1, 2, and 3 weeks on ibrutinib treatment), CXCR4 density (as measured on [68Ga]Pentixafor-PET) shifted from the bone marrow and lymph nodes toward the spleen. CXCR4+ CLL cells also decreased in the bone marrow. Ibrutinib-induced lymphocytosis presented with an increased fraction of CXCR4high and a loss of CXCR4low CLL cells. Because nonproliferating CLL cells in the bone marrow and the lymphatic tissue express more CXCR4 than proliferating CLL cells (21), ibrutinib-induced lymphocytosis consists mainly of quiescent (tissue-resident) CLL cells (6), with a more open and accessible CXCR4 gene locus (22). We interpret the decrease in spleen size in these 3 patients in parallel to the increased splenic CXCR4 density/ expression on PET, increased cell density on DWI, and increased CXCR4 blood cell count as indicating a shift of tissue-resident CLL cells to the orthotopic splenic cavernous system. In a patient who did not demonstrate ibrutinib-induced leukocytosis at short-term follow-up (2 weeks), no increased CXCR4 density in the spleen was visible on [68Ga]Pentixafor-PET, in line with our hypothesis.

The proposed compartment shift observed on [68Ga]Pentixafor-PET/MRI at short-term follow-up was still present at mid-term follow-up (2–4 months). However, concomitant leukocytosis was only partly present (11-week follow-ups, but not at the 18-week) reflecting a turning point in the course of disease. Our interpretation was also supported by a decrease in cell density as indicated by increased ADCmean.

Long-term follow-up [68Ga]Pentixafor-PET/MRI found that CXCR4 and cell density in the spleen decreased and splenic volume was reduced, possibly indicating elimination of quiescent CXCR4high CLL cells. Bone marrow CXCR4 density and lymph node
Figure 2.
Loss of CLL cell viability and increase of CXCR4 surface expression under ibrutinib therapy in patients 1 to 3. Left, flow cytometry of the 3 patients before ibrutinib and on ibrutinib treatment depicts a gain of DAPI^+ cells that suggests an increase of dead and apoptotic cells, whereas CXCR4 expression increases. Right, quantification of the percentages of DAPI^+ and CXCR4^+ cells of indicated patients.

Figure 3.
scRNA-seq of CLL cells before and during ibrutinib therapy (GSE109085). A, PCA of transcriptomic data from single CLL cells reveals distinct clustering of cells from 3 patients. Each dot represents a single CLL cell, color-coded to display CXCR4 expression. B, Average CXCR4 expression calculated from the clusters displayed in A.
volume and cell density also decreased. Remaining lymph nodes showed no change in CXCR4 density. The CXCR4, cell density, and tissue volume dynamics over three time points confirm the ability of \(^{68}\text{Ga}\)Pentixafor-PET-based CXCR4 imaging to capture the mode of action of ibrutinib.

No functional or metabolic imaging test exists for leukemia, including CLL. Unlike most lymphomas, glucose metabolism in CLL lesions is typically low. Therefore, PET with \(^{18}\text{F}\)FDG is not indicated for treatment response assessment and is used only to exclude Richter transformation (7). CXCR4 imaging with \(^{68}\text{Ga}\)Pentixafor-PET, when combined with MRI (or CT) for morphologic lesion assessment, provides a functional diagnostic for CLL. We show here that this approach allows visualization of the mode of action of BTK inhibition in patients with CLL. PET/MRI is further enhanced by the use of DWI, an MRI technique for indirect assessment of cell density that has been previously used for treatment response assessment in lymphoma and myeloma (23, 24). In our case series, the reduced diffusivity in the spleen reflecting increased density on ibrutinib treatment further supports our interpretation that the increased \(^{68}\text{Ga}\)Pentixafor uptake in this organ was not simply caused by higher expression of CXCR4 on CLL cells, but, at least in part, also by an increase in the number of CLL cells. Elimination of CLL cells from the spleen in later treatment phases was evident from long-term follow-up using \(^{68}\text{Ga}\)Pentixafor-PET/MRI.

In conclusion, we here provide CLL cell tracking evidence ranging from 1 to 18 weeks of response to ibrutinib treatment. Our analyses confirmed an early shift of CXCR4\(^++\) CLL cells from lymph nodes to peripheral blood and also revealed that ibrutinib released CLL cells from the bone marrow within 1 week. CLL cells liberated from bone marrow and lymph nodes into the peripheral blood also redistributed to the orthotopic splenic cavernous system, which decreased in volume after the leukocytosis resolved, as shown by follow-up 6 weeks or later. Visualization of CLL in the context of ibrutinib treatment supports the preexisting clinical hypothesis of a “compartment shift.” Our visualization also modified and refined the mechanistic model by describing early and long-lasting redistribution of CLL cells from the bone marrow to the peripheral blood and the spleen. \(^{68}\text{Ga}\)Pentixafor-PET/MRI labeling and visualization thus tracks CLL cells in patients.

**Disclosure of Potential Conflicts of Interest**

M.E. Mayerhoefer reports personal fees from Bristol-Myers Squibb, and grants and personal fees from Siemens outside the submitted work. U. Jager reports personal fees from Janssen during the conduct of the study, as well as grants and personal fees from AbbVie, Celgene, Gilead, Novartis, Roche, and Takeda Millennium, and personal fees from Amgen, Sandoz, Sanofi, and Miltenyi Biotec outside the submitted work. H.-J. Wester reports a patent for PCT/EP2011/056358 issued, and with royalties paid from Scintomics GmbH/PentixaPharm GmbH and is a shareholder of Scintomics GmbH, which is a shareholder of PentixaPharm GmbH. L. Kazianka reports grants from Austrian Academy of Sciences (OeAW) during the conduct of the study and grants from Comprehensive Cancer Center (CCC) Vienna outside the submitted work. P.B. Staber reports personal fees from Janssen outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

M.E. Mayerhoefer: Conceptualization, data curation, writing–original draft. A. Haug: Resources, investigation. U. Jager: Conceptualization, resources. V. Pichler: Investigation. S. Pfaff: Investigation, methodology. H.-J. Wester: Methodology. M. Hacker: Resources. I. Kazianka: Data curation, formal analysis, investigation, visualization. P.B. Staber: Conceptualization, supervision, writing–original draft, project administration, writing–review and editing.

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