Possible involvement of tyrosine kinase inhibitors on the expression of *CXCR4* in chronic myeloid leukemia

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Original Article

Abstract

Purpose: Chronic myeloid leukemia (CML) treatment has improved significantly in the last decade with the introduction of tyrosine kinase inhibitors (TKIs), which target BCR/ABL oncoprotein. However, a large proportion of patients develop resistance to this treatment protocol, with frequent relapse cases. CXCR4 up-regulation in leukemic cells induced by TKIs has been reported as a mechanism of chemoresistance by promoting migration of these cells to bone marrow, where they receive pro-survival signals and persist as quiescent cells. In the present study, we investigated the possible influence of treatment on the expression of *CXCR4* and *CXCL12* in peripheral blood cells of CML patients. **Methods:** Relative expressions (RE) were calculated from mRNA obtained from leukocytes of 21 patients in chronic phase, under treatment, and 54 healthy individuals, used as controls. **Results:** *CXCR4* expression was increased in CML patients compared to controls (RE: 1.931; p = 0.006). In CML patients, *CXCR4* and *CXCL12* expressions were correlated (r = 0.631; p = 0.002), and no differences in the expression of these genes were observed among different treatment protocols. However, *CXCR4* expression was positively correlated with imatinib treatment period duration of treatment (r = 0.56; p = 0.02). **Conclusion:** This data has pointed peripheral blood *CXCR4* expression as a possible marker for treatment monitoring, which may be useful to predict which patients would be benefit from newly developed treatments targeting CXCR4 and BCR/ABL concomitantly.

Keywords: Chronic Myeloid Leukemia; Tyrosine Kinase Inhibitors; CXCL12; CXCR4

Introduction

Chronic myeloid leukemia (CML) is a hematological disease that represents 20% of all adult leukemias, with an annual incidence of 1-2 cases per 100,000 individuals. This disease occurs at any age, but peaks of incidence are seen at 5th and 6th decades of life.[1,2] The prevalence of CML is increasing worldwide, and in 2014 estimates indicated 160,000 people living with this disease.[3]

CML is a proliferative disease of the hematopoietic system characterized by an overproduction of granulocytic lineage cells, and is associated with translocation between chromosomes 9 and 22, generating the fusion protein BCR-ABL.[4] This fusion gene results in constitutive activation of the ABL kinase, whose oncogenic properties include inhibition of apoptosis, proliferative advantage independent of growth factors, altered cell-cell and cell-matrix interactions and leukemogenesis.[5]

Historically, CML patients were treated with hydroxyurea and interferon-alpha (IFN-α) alone or in combination with low-dose cytarabine, and allogeneic stem cell transplantation. CML treatment changed dramatically in 2001 with the approval of imatinib, the first BCR-ABL tyrosine kinase inhibitor (TKI), followed by dasatinib, nilotinib, bosutinib, and more recently ponatinib.[6]

Standard treatment of CML with TKIs is highly effective in reducing disease burden, delaying disease progression, and prolonging overall survival of patients. However, up to 20% of CML patients who start on first-line TKI therapy will develop resistance to treatment which is caused, mainly, by mutations in the *BCR-ABL* kinase domain, hampering TKI-binding sites.[7]

CXCR4 is a seven-transmembrane G-protein-coupled chemokine receptor, expressed in hematopoietic and

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epithelial cancer cells [8], and appear to be an essential factor for survival and chemotherapy resistance in leukemia. CXCL12, a ligand for CXCR4, is a soluble pro-survival factor secreted by bone marrow (BM) stromal cells, the primary component of BM microenvironment.[9,10] The binding of CXCL12 to CXCR4 induces several intracellular signals related to chemotaxis, homing, cell survival and proliferation, and is involved in treatment resistance of CML.[9]

Apart from its role in the pathogenesis of CML, CXCR4/CXCL12 axis regulates stem/progenitor cell migration and retention in the marrow, and is required for hematopoiesis. In 2002, Ptasznik, Urbanowska[11] demonstrated for the first time, the existence of a crosstalk between BCR-ABL and CXCR4 pathways in leukemia cells: BCR-ABL disrupted chemokine signaling and chemotaxis, and increased the ability of immature myeloid cells to escape from BM into circulation. However, studies have shown that the downregulation of CXCR4 expression could be reversible by TKIs, such as imatinib, promoting migration of CML cells to BM stroma, causing cycle arrest and, hence, ensuring the survival of quiescent CML progenitor cells.[12,13]

In this context, the present study aimed to investigate the expression of *CXCL12* and *CXCR4* in peripheral blood cells of CML patients and healthy individuals, and the impact of TKI treatment on their expressions.

Methods and Materials

Study population
Following approval from the Human Ethics Committee of the State University of Londrina (CAAE n°. 0164.0.268.000-09) 5 mL of peripheral blood were obtained from 21 patients with clinical and hematological diagnosis for CML, in the chronic phase and under treatment at the time of sampling. All patients and healthy individuals (n = 54) were attended in the Cancer Hospital of Londrina and University Hospital of State University of Londrina, Londrina-PR, Brazil, and have signed a term of free informed consent.

RNA extraction and cDNA synthesis
Leukocytes were prepared from peripheral blood samples using red blood cells (RBC) lysis buffer (SigmaAldrich). Total cellular RNA was obtained from these cells submitted to TRIzol LS reagent extraction (Invitrogen™, Carlsbad, U.S.A.) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically at 260 and 280 nm, andstored at -20° C until use. Reverse transcription reaction (RT) was performed using 500 ng of RNA, 20 units of cloned moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen™) and 4 units of recombinant ribonuclease inhibitor (RNaseOUT™; Invitrogen™), under the following conditions: 2.5 μM oligo dT, 50 mM Tris HCl pH 8.3, 75mM KCl, 1.5 mM $MgCl_2$, 1.25 mM of dNTP, at 42° C for 60 min in Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada).

Molecular analysis of *Beta-actin* mRNA
PCR for *beta-actin* amplification was performed with cDNA to analyze the viability of the RNA samples and cDNA quality. Briefly, cDNA synthesis was carried as described by Amarante *et al.*[14], and PCR conditions were: 94°C for 1 min followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and finally, 72°C for 10 min in Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada). The amplified PCR product consisted of a 353bp fragment, analyzed in a polyacrylamide gel (10%) electrophoresis. All the RNA samples presented detectable quantities of beta-actin mRNA and acceptable integrity during amplification. No contamination with genomic DNA was verified.

Quantitative real-time PCR for CXCL12 and CXCR4 mRNA
Real-time PCR using SYBR green fluorescent dye was performed with 2.0μl cDNA in 20μl total reaction volume. Each real-time PCR reaction consisted of 2.5μL RT-PCR product, 10μL Platinum®SYBR Green qPCR SuperMix UDG (Invitrogen ™) and 0.25μM of each sense and antisense primer.

For quantitative polymerase chain reaction (qPCR) was used specific primers: 5' TTACCCGCA AAAGACAAGT 3' as sense and 5' AGGCAATCACAAAACCCAGT 3' as antisense primers for *CXCL12*, and 5' TGTTGGCTGAAAAGGTGGTC 3' as sense and 5' AAA GATGAAGTCGGGAATAGTC 3' as antisense primers for *CXCR4*. As a housekeeping gene, *human glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* was amplified using the sense primer 5' GAAGGTGAAGGTCGGA 3' and antisense primer 5' GGGTCATTGATGGCAAC 3'. The PCR reaction was performed for 40 cycles as follows: 95°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec in a Chromo4™ Real Time PCR Detection (Bio-Rad, Hercules, U.S.A.). The qPCR results were analyzed according to the Pfaffl method.[15]

Statistical Analysis
Differences for gene expression between CML and control group were assessed using the relative expression software tool (REST) 2009. Nonparametric Kruskal-Wallis and Mann-Whitney U tests were performed to compare *CXCL12* and *CXCR4* expressions in groups treated with different drugs (imatinib, nilotinib or other) and different imatinib doses (400 or 600-800 mg/day). Correlation analyses were performed using Spearman's nonparametric test. Data were analyzed using GraphPad Prism 6.0, with the level of significance set at $p < 0.05$.

Results

Sample characterization

Twenty-one patients with CML under treatment were included: 15 were treated with imatinib (9 patients with 400mg/day; 5 with 600mg/day and 1 with 800mg/day), 3 with nilotinib (200mg/day), 2 with dasatinib (70mg/day and 200mg/day) and 1 with hydroxyurea (1g/day).

Median age was 44.50 years (range: 31-80) in CML group and 36.00 years (range: 25-56) in control group. Despite this difference being statistically significant (p = 0.0004), no influence of age in the expression of *CXC12* or *CXCR4* was detected either in control (p = 0.61, r=0.07 and p = 0.66, r = -0.06 for *CXCL12* and *CXCR4*, respectively) or in CML group (p = 0.68, r = 0.09 and p = 0.51, r = 0.14).

CXCR4 but not *CXCL12* is differentially expressed in CML patients

CXCL12 was not differently expressed in CML patients compared to control group (fold change expression: 0.960; p = 0.82). However, *CXCR4* expression was approximately two-fold higher (RE: 1.80) in patients with CML compared to controls (p = 0.02). **Figure 1** shows the expression distribution of each CML patient in relation to control group.
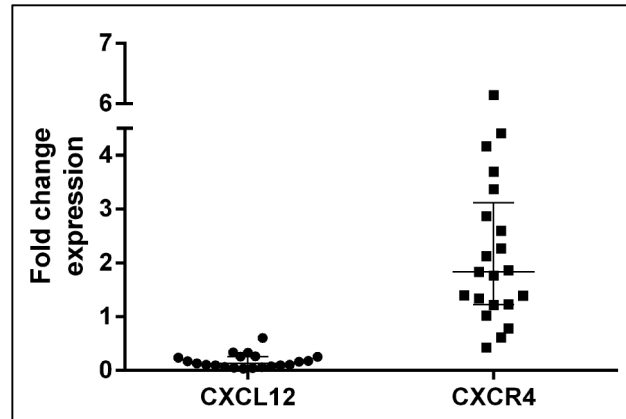

FIG. 1: *CXCL12* and *CXCR4* relative expressions in CML patients. Individual values were calculated using mean cycle thresholds and efficiency values as parameters. Lines indicate median expression value plus interquartile range.

CXCL12 and *CXCR4* expressions are correlated in CML patients, but not in controls

We investigated if there was a correlation between *CXCL12* and *CXCR4* expression in leukocytes, and our results showed that these genes were positively correlated in CML (n = 22, p = 0.002; r = 0.631) but not in control group (n = 54, p = 0.68; r = 0.06) (**Figure 2**).
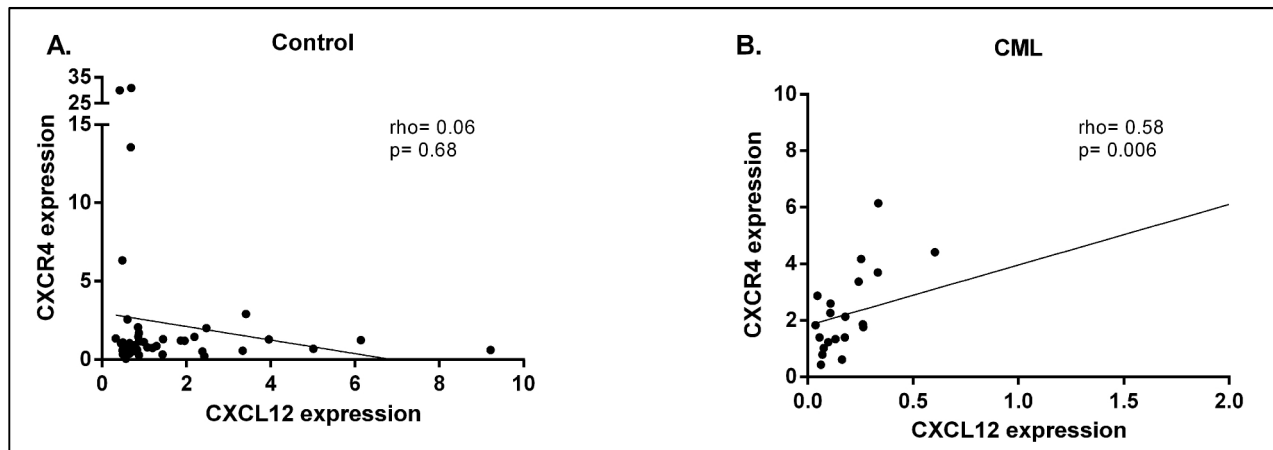

FIG. 2: *CXCL12* and *CXCR4* expressions are correlated in CML. Correlation analyses between *CXCL12* and *CXCR4* relative expressions in controls (A) and CML (B) groups. Data are shown as individual expression values relative to mean control expression. P and rho values were obtained from Spearman's correlation tests.

CXCL12 and *CXCR4* expressions between different treatment protocols among CML

Peripheral blood *CXCL12* and *CXCR4* expressions were compared between patients receiving imatinib (n = 15), nilotinib (n = 3) or other treatment (2 patients receiving dasatinib and 1 receiving hydroxyurea). No significant differences between these treatment groups were observed for any of the studied genes (p = 0.972 for *CXCL12* and p = 0.542 for *CXCR4*) (**Figure 3A and 3B**).

In patients undergoing treatment with imatinib, *CXCL12* and *CXCR4* expressions were compared between those receiving 400 mg and 600 – 800 mg of the drug per day. No statistical differences were observed for the expression of both genes (*CXCL12:* p = 0.14; *CXCR4:* p = 0.17) (**Figure 3C and 3D**). These results suggest that the observed increment in CXCR4 expression in CML patients is not dependent on treatment type or dose.
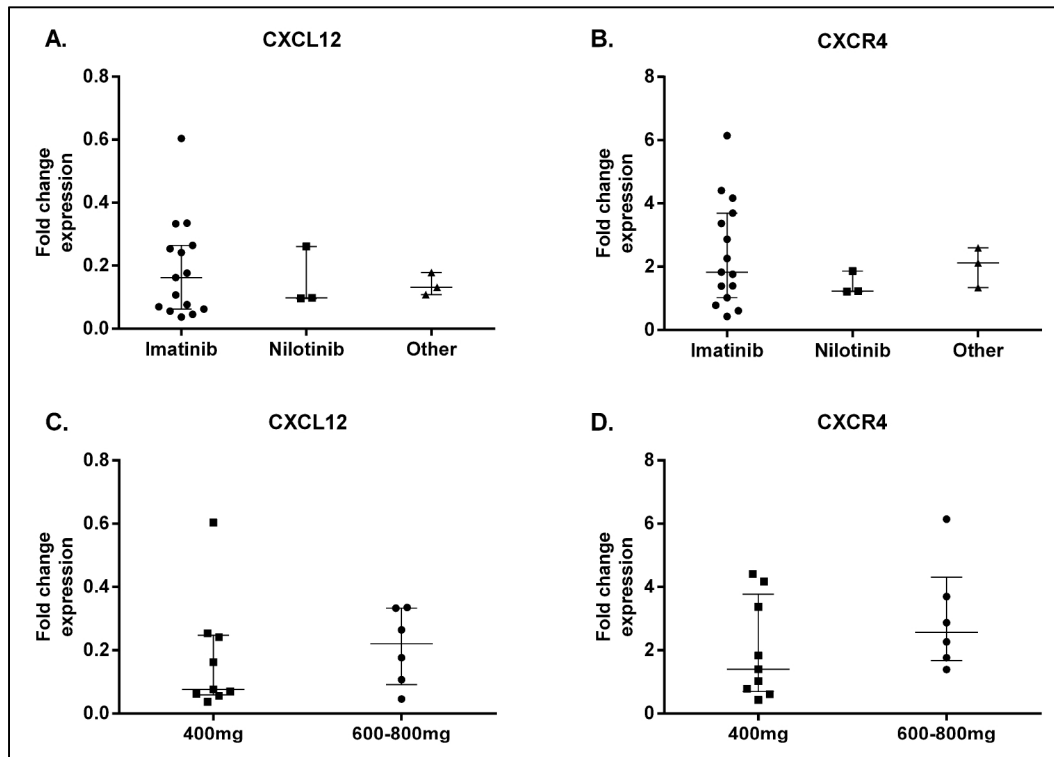
FIG. 3: *CXCL12* and *CXCR4* expressions according to treatment drug and imatinib dose. *CXCL12* (A) and *CXCR4* (B) relative expressions were compared between patients receiving different treatment protocols. The group "other" was composed by two patients receiving desatinib and one receiving hydroxyurea. (C and D): *CXCL12* (C) and *CXCR4* (D) expression comparison between patients receiving 400 or 600-800mg. Lines indicate median expression value plus interquartile range.
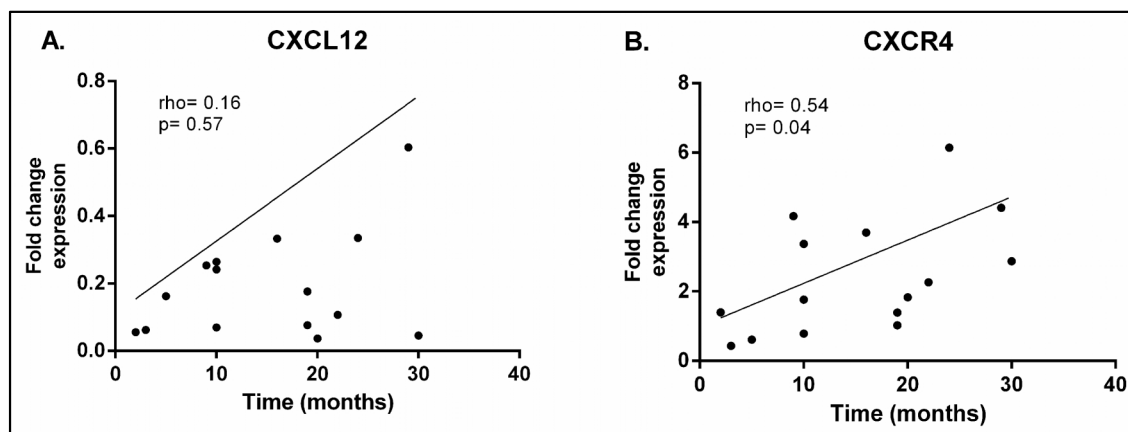

FIG. 4: *CXCL12* and *CXCR4* expression in relation to the treatment time in CML patients. Correlation analyses between expressions of *CXCL12* (A) and *CXCR4* (B) and treatment duration. P and rho values were obtained from Spearman's correlation tests.

CXCR4 expression was positively correlated with treatment time with imatinib

We investigated weather treatment time with TKI influenced *CXCL12* or *CXCR4* expression in CML patients. For this purpose, we performed correlation analyses between treatment time and either *CXCL12* and *CXCR4* expressions only in patients that were being treated with imatinib (n = 15). It was observed a positive correlation between *CXCR4*

expression and treatment time with imatinib ($p = 0.02$; r = 0.56) (**Figure 4A**). No correlation was observed for *CXCL12* expression ($p = 0.44$; r = 0.21) (**Figure 4B**).

Discussion

CML is a malignant neoplasia of hematopoietic stem cells, resulting in massive presentation of myeloid lineage cells in

peripheral blood and excessive myeloid proliferation in the BM[16], usually affecting people over 45 years. In this study, CML patients had ranged in age from 31 to 80 years (median: 44.5 years), which is in agreement with Cid, Magalhaes[17] that found a median age CML group consisting of 41.5 years. According to another study realized in 20 European countries with CML patients, the median age found was 55 years[18]; but it is considerably lower in countries where the population is younger.[19]

Chemokines and their receptors are involved in the development of several disorders, including cancer. Particularly, the chemokine receptor CXCR4 has been reported to be highly expressed in cancer cells, including those from hematopoietic origin, and essentially implicated in leukemia cell survival and drug resistance.[20,21] Moreover, CXCR4/CXCL12 interaction activates several intracellular signals related to chemotaxis, homing, cell survival and proliferation.[22]

Accumulating evidence suggests that leukemia cells are protected by marrow stromal cells (MSCs) [23] and indirect communication through CXCL12 and direct contact between leukemia cells and MSCs have been demonstrated to be essential for CML cell survival and resistance.[24] CXCL12 may protect CML cells from chemotherapeutic-induced apoptosis, and activation of CXCR4 induces CML cell homing to the BM microenvironment.[20] Our study demonstrated that *CXCR4* expression was higher in peripheral blood of CML patients when compared to healthy controls and correlates with *CXCL12* expression. These data corroborate with several studies reporting important involvement of the *CXCR4/CXCL12* axis as a potential target for new therapeutic strategies in leukemia patients.[25,26]

CML was the first cancer in which an effective targeted-therapy has been outlined [27], and once TKIs were developed, they were proved to be more efficient in controlling the disease than other types of chemotherapy, such as IFN-α or hydroxyurea. This therapy targets the tyrosine kinase BCR/ABL, which is responsible of leukemogenic events in CML, and the use of these inhibitors can reverse the malignant changes in the leukemic cells and/or cause cell apoptosis.[28,29]

Imatinib is effective in most CML patients, however a considerable proportion of them, some still in the chronic phase and a higher proportion in later phases, are resistant or intolerant to imatinib.[30,31] It is known that some patients fail initial treatment (primary resistance), while others lose a previously acquired response (secondary resistance); the latter is the most common and associated with development of mutations in the BCR/ABL site.[32,33] Other tyrosine kinase inhibitors were developed as second generation drugs, such as nilotinib and dasatinib[34], but they both might induce resistance.[35,36]

Several other strategies are in use to improve CML treatment, including imatinib dose escalation combining different therapies, initial use of second generation tyrosine kinase inhibitors and maintaining therapy with IFN-α and vaccine.[37]

The resistance mechanisms have been analyzed and the most common is reactivation of kinase activity by BCR/ABL point mutations or gene amplification.[38] A point mutation in the tyrosine kinase binding site can prevent imatinib coupling by interrupting critical points of contact, or by inducing a protein impairment to which the drug cannot associate.[39] Depending on the mutation present, dose escalation may be a good strategy to restore the response to imatinib.

More recently, analysis of gene expression profiles identified the expression of the chemokine CXCL12 as a sensitivity predictor to imatinib. Vianello, Villanova[10] reported that leukemic cell survival mechanism in CML mediated by BM stroma mesenchymal cells is related to protection from apoptosis induced by imatinib, through CXCR4/CXCL12 activation. In the present study we verified that *CXCR4*, but not *CXCL12*, expression is positively correlated with imatinib treatment time.

It has been demonstrated that imatinib treatment increased *CXCR4* expression and induced leukemic cell migration to BM stroma, which promoted survival of quiescent CML cells during treatment[13], making approaches targeting both BCR-ABL and CXCR4 attractive to more efficiently manage disease and overcome treatment resistance.

Otherwise, Peled, Hardan [40] demonstrated that CD34+ Philadelphia-positive CML cells expressing CXCR4 migrate in response to factors derived from BM stroma (particularly, CXCL12). However, CD34+ CXCR4+, but Philadelphia negative (normal) cells from the same patient showed higher rates of migration by CXCL12, indicating a functional role mediated by CXCL12 in retention of CD34+ immature cells and progenitor cells in BM of healthy individuals and CML patients.

Moreover, it was recently shown that CXCR4 overexpression in the CML cell line K562 does not increase the migratory potential of CML cells, but rather supports their survival and growth, therefore confirming the pro survival role of CXCR4 in CML.[41]

Though, it is reasonable to assume that inhibiting CXCR4 might represent a possible therapeutic strategy in managing CML patients experiencing drug resistance. In fact, Zeng, Samudio[42] described that CXCR4 inhibition with the novel RCP168 peptide overcomes stroma-mediated chemoresistance in chronic and acute leukemias. In the same way, another high affinity CXCR4 inhibitor, BKT140, abrogated proliferation and induced significant apoptotic cell death in CML cells, mainly in combination with imatinib.[41]

Similarly, plerixaflor (AMD3100, Genzyme Corporation), a CXCR4 inhibitor used for cell mobilization from BM to peripheral blood in hematopoietic stem cell transplantation, was shown to disrupt the interaction of CML cells with BM, sensitizing them to nilotinib treatment. Co-administration of plerixaflor and nilotinib delayed time to relapse and prolonged survival in a xenograft model of CML in mice.[43] Co-inhibition of BCR-ABL and CXCR4 already reached a phase I clinical trial (identifier NCT02115672 at https://clinicaltrials.gov website), but are not yet open for patient recruitment.

Several mechanisms should be considered in treatment failure with imatinib, and they should be clarified to offer patient the best second-line treatment, whether dose optimization, another tyrosine kinase inhibitor or BM transplantation. In future, multiple treatment options will be available to CML patients and strategic combinations of new anticancer drugs, taking into account the patient's condition and response to different drugs, might prevent the development of resistant clones.

Finally, time of treatment with imatinib was positively correlated with *CXCR4* relative expression in CML patients, suggesting that *CXCR4* up-regulation by imatinib occurs even in peripheral blood cells. As these cells are readily accessible, our results might indicate a feasible way to monitor *CXCR4* expression during TKI therapies, which may have a direct prognostic role indicating which patients are more likely to develop chemoresistance and which of them might be benefited by co-treatments with TKI and CXCR4 inhibitors. Future cohort studies investigating the association between *CXCR4* expression in peripheral blood leukocytes and relapses or TKI resistance in CML patients will be necessary to corroborate this analysis as a feasible method for clinical use in treatment monitoring.

Conflict of interest

The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgment

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