Peripheral blood CD26 positive leukemic stem cells as a possible diagnostic and prognostic marker in chronic myeloid leukemia

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ARTICLE INFO

Keywords:
CP-CML
CD26
LSCs
TKI

ABSTRACT

Background: CD26 is expressed in all chronic myeloid leukemia (CML) patients. This study investigated the role of CD26+ LSCs in diagnosis and follow up of CML patients.

Method: Flow cytometry was performed to evaluate CD26+ LSC in peripheral blood (PB) in CML patients. BCR-ABL1 transcript level measurement was performed using standard qRT-PCR technique.

Results: CD26+ LSCs were significantly correlated with BCR-ABL1 transcript level at diagnosis and after three months of treatment. CD26+ LSCs also were significantly associated with the risk score after 12 months of treatment.

Conclusion: CD26+ LSCs can be a useful marker in diagnosis and follow up of patients with CML.

Abbreviations
CML: chronic myeloid leukemia;
TKI: tyrosine kinase inhibitors;
LSCs: leukemic stem cells;
qRT-PCR: quantitative real-time-polymerase chain reaction;
BM: bone marrow;
PB: peripheral blood;
HSCs: hematopoietic stem cells;
TFR: treatment-free remission;
DMR: deep molecular response;
ELN: European LeukemiaNet;
MMR: major molecular response;
SD: standard deviation

1. Introduction

Hematopoietic stem cells (HSCs) are multipotent cells that are generated via progenitor cells of all blood lineages and reside in the bone marrow (BM) niche, which regulates HSC survival and function. Leukemic stem cells (LSCs) are responsible for disease initiation and progression. They share many characteristics with normal HSCs, including quiescence, multipotency, and self-renewal [1]. The identification of LSCs and their separation from normal HSCs in chronic myeloid leukemia (CML) are challenging since both populations reside in the same compartment that is phenotypically defined as CD45-34+38-. Moreover, little is known about the function and phenotype of LSCs in CML or about specific markers that distinguish them from HSCs [3]. CML treatment has been revolutionized by the introduction of specific BCR-ABL1 tyrosine kinase inhibitors (TKI) such as imatinib, nilotinib, and dasatinib, which induce apoptosis in leukemic cells. The response to TKI is heterogeneous, and approximately 40% of patients may need a switch of TKI due to intolerance or a poor response [3,4]. Though a subset of TKI-treated CML patients can achieve a deep molecular response (DMR) during therapy, only half of them (or even less) can sustain a treatment-free remission (TFR). However, some patients with optimal response to TKI may have residual diseases that originate from TKI-resistant quiescent LSCs [4].

It is difficult to predict relapse. Moreover, approximately 40%-60% of DMR patients who stop taking TKIs will lose their response and
require retreatment [5] while others will maintain TFR even with detectable molecular disease in some cases [5,6]. Relapse after TKI discontinuation may be due to the persistence of LSCs surviving TKIs through the activation of several BCR-ABL1-independent pathways [7]. Quantitative real-time–polymerase chain reaction (qRT-PCR) may be inappropriate to quantify residual quiescent CML LSCs, which may be transcriptionally lower silent while surviving indefinitely into tumor-specific hypoxic niches [8]. Different biomarkers have been tested to better distinguish the leukemic clones from their normal counterparts. Herrmann et al. [2] reported that CD26 (dipeptidylpeptidase IV) is a potential biomarker for the quantification and isolation of CML LSCs in BM samples of CML patients. In contrast to other tested antigens such as CD90 and IL-1RAP that are co-expressed on CML LSCs, acute myeloid leukemia LSCs, and normal HSC, CD26 was the only marker expressed in all tested CP-CML patients, which was not present on CD34+ LSCs of normal BM or on LSCs of other myeloid neoplasms [9]. CD26 can induce the degradation of cytokine ligands such as SDF-1, a molecule that normally attracts HSCs and LSCs into the BM niche via the CXCR4 receptor [10]. This degradation facilitates LSC mobilization from the BM niche, suggesting that CD26 could have a fundamental role in the regulation of LSC niche interaction and help LSCs escape TKI treatment [2]. BM CD26+ LSCs are characterized by the expression of BCR-ABL1 transcripts and their numbers appear to decrease substantially during successful treatment with TKIs, which indicates that CD26 could also be a good predictive biomarker for MRD monitoring of CML patients during therapy [11] and that flow cytometry approach could be a useful tool for the identification of CML LSCs on BM samples by using a CD45+/CD34+)/CD38- /lowCD45RA–cKIT–CD26+ phenotype. Raspadori et al. [14] confirmed the appropriateness to detect and quantify LSCs directly from peripheral blood (PB) samples of CML. Raspadori et al. [14] confirmed the appropriateness to detect and quantify LSCs directly from peripheral blood (PB) samples of CML. Understanding the heterogeneity of the LSC compartment is crucial for preventing treatment failure [15]. The exact role of CD26 in CML patients on TKI therapy is still unknown.

This prospective study aimed to evaluate CD26+ LSCs in PB by flow cytometry in CML patients at diagnosis and during TKI treatment in a bid to correlate them with a molecular response.

## 2. Patients and methods

### 2.1. Patients

This study was carried out on 48 CP-CML patients from January 2019 to December 2021. It included 48 newly-diagnosed CP-CML patients (23 males and 25 females) aged 34–69 years with a mean age of 49.8±6.91 years. All participants gave their written informed consent to participate in the study, which was approved by the ethical committee of Zagazig University. Patients were diagnosed according to the WHO diagnostic criteria (2008). This prospective study was carried out in Internal Medicine, Clinical Pathology, and Medical Biochemistry & Molecular Biology Departments of Zagazig University. All patients started TKI treatment (Imatinib), and follow-up was performed after three, six, and twelve months. We excluded CML patients under other types of TKIs and those other than CP-CML phase.

## 3. Methods

### 3.1. Sample preparation

Five-milliliter EDTA PB samples were collected and analyzed within 24 h. CD26 expression was evaluated via standardized multiparametric flow cytometry, and the CD45+/CD34+/CD38+ population was analyzed using the eight-color staining protocol via the lyse stain wash procedure. Red cell lysis was performed with BD (Ref 349,202, BD Biosciences, San Jose, CA) diluted in a 1:10 ratio in deionized water. After lysis, 2.0×10⁶ leucocytes/ml were incubated with 5 µL of a monoclonal antibody (CD45 V-500 BD clone H130 BD), (CD34 PerCP BD clone 8G12 BD), (CD38 APC BD Clone HB7), and (CD26 PE BD clone L272). Internal negative control staining samples were used, and events were acquired and performed with the FACS Canto II flow cytometer using the DIVA 8 software program (BD, Biosciences).

The instrument setup was monitored daily. To ensure reproducible results over time, a standardized protocol was implemented. It implied adjustments of FACS internal parameters, using the BD FACS Diva™ Cytognostic (Ref 656,505; BD Biosciences, San Jose, CA), to maintain the instrument performance by correcting the use of lasers and fluidic instability.

### 3.2. Flow cytometry

PB CD26+ LSC evaluations in CML patients were performed via a sequential gate at diagnosis. Cells of the CD45+ PE/CD34+ FITC/CD38 PerCP population were identified. FSC and SSC light properties to exclude debris and doublets, CD45/SCC was performed on viable cells and then gate on CD34/SSC cells to identify CD34+/CD38+ /CD45+ and CD45+/34+/38+ compartments, with the latter being referred to as the “LSCs.” The investigated CD26+ and CD26- SC populations were well identifiable. The absolute number of CD26+ LSC in PB samples was calculated as follows: (WBC count/µL) × (ratio of CD45+/CD34+/CD38-/CD26+ cells (%)) and expressed as LSC/µL [16] as shown in Fig. 1.

### 3.3. RNA extraction and cDNA synthesis

Total RNA was extracted from PB on EDTA. In brief, the samples were collected and stored at room temperature, and extraction procedures were performed within 24 h of the sample collection to avoid RNA degradation. RBCs were lysed and the remaining cells were diluted in 1 mL QIAzol according to the manufacturer’s instructions (Qiagen, Germany). To assess the quality and the integrity of the harvested RNA, it was run through gel electrophoresis and measured via Nanodrop spectrophotometry (ND 1000-NanoDrop®). Then, the cDNA was synthesized using a high-capacity cDNA transcription kit from 1 µg RNA according to the manufacturer’s instructions (Applied Biosystem) with a total reaction volume of 20 µL. (10 µL containing 1 µg RNA, 2.0 µL 10X RT Buffer, 0.8 25X dNTP mix (100 mM), 2.0 µL 10X RT random primers, 1.0 µL multiscribe reverse transcriptase, 1.0 µL RNase inhibitors, and 3.2 µL nuclease-free water) in a MicroAmp™ fast 96-well reaction plate (Applied Biosystem) thermal cycler with cycling condition of: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min for enzyme deactivation. cDNA was diluted in a ratio of 1:5 and stored at −20 °C for future use.

### 3.4. Quantitative polymerase chain reaction (Q-PCR)

PB BCR-ABL1 transcript level measurement was performed using standard qRT-PCR technique according to European LeukemiaNet recommendations [17] using Taqman universal master mix in accordance to the manufacturer’s instructions (Applied Biosystem) with 2 µL of the cDNA in a Rotor-Gene Q 2 plex (Qiagen, Germany), with the following cycling condition: initial denaturation at 95 °C for 10 min and 50 cycles of (denaturation 95 °C for 20 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s), followed by the melting curve analysis. For the calculation, the ABL gene was used as a control and the BCR-ABL1/ABL ratio was also calculated. The total number of copies was estimated by plotting a standard curve with seven different dilutions using a linearized plasmid containing inserts of the examined genes [18]. Major molecular response (MMR) was considered a three-log reduction from this baseline value. MMR values were adjusted to an international scale using a conversion factor of 1.19 [19].
4. Statistical analysis

Data were analyzed using IBM SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA) and NCSS 11 for Windows (NCSS LCC., Kaysville, UT, USA). Quantitative data were expressed as the mean ± standard deviation for normally distributed data and as median values with inter-quartile ranges for variables with skewed data distributions. Qualitative data were expressed as frequencies and percentages. The independent-samples t-test and the Mann-Whitney U test were performed for normally distributed and non-normally distributed quantitative data, respectively, while the Chi-square test, Fischer exact test, and Pearson’s correlation coefficient were used to analyze qualitative data. P-values of <0.05 were considered statistically significant while P-values of <0.001 were considered highly significant.

5. Results

5.1. Demographic and basic characteristics of the CP-CML patients

Twenty-three patients (47.9%) were males and 25 patients (52.1%) were females. Their age ranged from 34 years to 69 years, with a mean value of 49.8 ± 6.91 years. The Sokal risk was intermediate in 23 patients (47.9%) and high in 25 patients (52.1%). The mean absolute number of CD26+ LSCs/µl at diagnosis was 21.3 ± 22.14 and the mean BCR-ABL1 ratio at diagnosis was 54.4 ± 18.8 (Table 1). Two patients were lost after three months and four were lost after 12 months.

5.2. Differences in CD26+ leukemic stem cells and BCR-ABL1 levels after treatment and on follow up

CD26 levels after three months and after 12 months of treatment were significantly lower than their values at diagnosis (P < 0.001 for both). No significant difference was found between CD26+ LSCs at three months and 12 months of treatment (Table 2).

BCR-ABL1 transcript level at diagnosis was significantly higher than that after three months of treatment and after 12 months of treatment (P < 0.001). BCR-ABL1 level after 12 months of treatment was significantly lower than its values after three months of treatment (P < 0.001, Table 3).

5.3. Correlations between CD26+ leukemic stem cells and BCR-ABL1 and other data of studied patients

There was a significant positive correlation between CD26+ LSCs

Table 1
Demographic and basic characteristics among CP-CML patients.

|                      | CP-CML cases N = 48 |
|----------------------|---------------------|
| Age(years)           | 49.8 ± 6.91         |
| HB (g/dL)            | 8.4 ± 1.63          |
| WBCs count (10^9 /L) | 137.5 ± 47.5        |
| Platelet count (10^9 /L) | 224.5 ± 54.5    |
| PB blast (%)         | 2.73 ± 0.91         |
| Spleen size (cm/below CM) | 12.71 ± 4.3   |
| Absolute number of CD 26+ LSCs/µl at diagnosis | 21.3 ± 22.14    |
| BCR-ABL1 transcript level at diagnosis | 54.4 ± 18.8 |
| Gender               |                     |
| Male                 | 23                  |
| female               | 25                  |
| Sokal risk           |                     |
| Intermediate         | 23                  |
| High                 | 25                  |

Fig. 1. Peripheral blood (PB) CD26+ leukemic stem cells (LSCs) flow cytometry evaluation in chronic myeloid leukemia (CML) patients: (A) CD45 versus SSC; (B) Gate CD34+ cells; (C) CD34+/CD38+/CD26+ LSCs and CD34+ / CD38+ populations; (D) CD26 expression on CD34+ CD38+ gated cells (95.5%).
and BCR-ABL1 at diagnosis ($P = 0.006$) and after three months of treatment ($P = 0.03$) but there was no statistically significant correlation between CD26$^+$ LSCs and BCR-ABL1 after 12 months of treatment. There was a significant positive correlation between CD26$^+$ LSC counts and WBC counts at diagnosis, after three months, and after 12 months of treatment ($P = 0.03$, 0.001, and 0.007, respectively (Table 4, Fig. 2). Besides, there was a statistically significant positive correlation between BCR-ABL and WBC counts after three months of treatment ($P = 0.04$) and with platelet counts ($P = 0.002$, Table 5). Moreover, there was a statistically significant difference in CD26$^+$ LSCs at 12 months after treatment between patients with high Sokal risk scores and those with intermediate risk ($P = 0.01$, Table 6).

### 6. Discussion

**BCR-ABL1 oncogene targeted treatment with TKI showed impressive efficacy** against proliferating CML cells; however, the rapid relapses in more than half of the CML patients after treatment withdrawal suggest the presence of quiescent LSCs that are inherently resistant to BCR-ABL1 inhibition [20]. Most insensitive TKI cells of the BM LSCs compartment are defined by the CD34$^+$ CD38$^-$ /low CD45$^-$ CD26$^+$ phenotype, which suggests the crucial role of CD26$^+$ LSCs [14]. However, the phenotypes and molecular signatures of treatment-resistant BCR-ABL1 positive stem cells are yet to be established [10]. Well-defined eligibility criteria to predict safe discontinuation are still to be established [15]. The crucial role of CD26$^+$ LSCs in the follow-up of CML during TKI treatment is not well understood. In this prospective study, we tried to quantify CD26$^+$ LSCs in CP-CML at diagnosis and during TKI treatment. We found that there was a statistically significant reduction in CD26$^+$ LSCs during treatment follow-up as they were detected in 100% of circulating leukemic cells at diagnosis, in 71.7% after three months of TKI treatment, and in 64.2% after 12 months of treatment. The presence of CD26$^+$ LSCs in 100% of our CP-CML patients was supported by the findings of Raspadori et al. [14] who reported similar results, which supports the usefulness of CD26 as a diagnostic marker for CML. This reduction in circulating CD26$^+$ LSCs after TKI treatment of CML patients was supported by the results of a previous study by Galimberti et al. [21]. So, we found that circulating CD26$^+$ LSCs were still present in the PB even after one year of successful TKI treatment. This may be due to the presence of quiescent leukemic cells that are resistant to TKI treatment and may be reactivated after treatment withdrawal, leading to disease recurrence [21]. Valenta et al. [10] reported that CD26$^+$ LSCs decreased rapidly after 3-6 months in patients with a molecular response and were no longer detected. In contrast, in relapsed or resistant CML patients, CD26$^+$ LSCs remain detectable. In line with our findings, Boccia et al. [15] found in their cross-sectional study that CD26$^+$ LSCs were detected in 100% of CML patients at diagnosis, 71.6% in patients under TKI treatment, and in 61% of patients with TFR who discontinued treatment. Herrmann et al. [2] found that expression of CD26 on CML LSC was not inhibited by the addition of imatinib. They suggested that CD26 expression may depend on other factors than BCR-ABL1 as the turnover of CD26 on the surface of CML LSC is low, so low that the inhibition of BCR-ABL1 was ineffective. They suggested the use of CD26 as a follow-up marker in patients under TKI treatment. Our results revealed that BCR-ABL1 was detected in all CP-CML patients at diagnosis, 89.1% after three months of treatment, and 88% after 12 months of treatment. This result was supported by the findings of Boccia et al. [12], who reported that 25 patients out of 108 had undetectable BCR-ABL1 transcript levels after imatinib treatment. This reduction in BCR-ABL1 with TKI treatment is supported by Herrmann et al. [2] who reported that there was a substantial decrease in BCR-ABL1 transcript levels to undetectable or near-undetectable levels after treatment. We also found that BCR-ABL1 transcript levels at diagnosis were significantly correlated with CD26$^+$ LSC counts, as well as after three months of treatment; however, BCR-ABL1 transcript levels were not correlated with CD26$^+$ LSC counts after one year of treatment. On the other hand, Valent et al. [10] reported that LSC assays are not sensitive compared to BCR-ABL1 transcript levels monitoring, even when analyzing larger cell numbers in CML. They also reported that the numbers of residual CD26$^+$ LSCs must be expected to correlate with the numbers of BCR/ABL molecules. So, they do not recommend the use of the CD26 LSC assay for routine follow-up monitoring in CP-CML. The absence of a correlation between BCR-ABL1 transcript levels and CD26$^+$ LSC counts at the start of treatment is supported by Boccia et al. [12] who found no correlation between residual CD26$^+$ LSCs in PB and the molecular response when they were compared in CML patients under TKI treatment for a median period of 68 months. We also detected that BCR-ABL1 transcript levels were significantly correlated with WBC counts at three months of treatment. We also found a statistically significant correlation between CD26$^+$ LSC and WBC counts. This result was in line with those of a study by Culen et al. [11] who reported that high WBC counts were associated with higher CD26$^+$ LSC counts. We detected a statistically

### Table 2

|                  | N | Median (range) | Friedman test | $P$  |
|------------------|---|----------------|---------------|-----|
| **Absolute number of CD26$^+$ LSCs/μL at diagnosis** | 48 | 19.8 (± 21.2) | <0.001** |
| **Absolute number of CD26$^+$ LSCs/μL after 3 months** | 46 | 0.026 (± 0.092) | 0.011 (± 0.06) |
| **Absolute number of CD26$^+$ LSCs/μL after 12 months** | 42 | 0.012 (± 0.014) | 0.011 (± 0.06) |

*P-value < 0.001 is highly significant; a: significant difference from level after 3 months; b: significant difference from level after 12 months; c: no significant difference between levels at 3- and 12-months using Wilcoxon test.

### Table 3

|                  | Mean ± SD (range) | Friedman test | $P$  |
|------------------|-------------------|---------------|-----|
| **BCR-ABL1 transcript level at diagnosis** | 48 | 55.3 (± 19.5) | <0.001** |
| **Median (range)** | 53.3 (5.1 – 99.7)$^ab$ |
| **BCR-ABL1 transcript level after 3 months** | 46 | 9.22 ± 2.7 |
| **Median (range)** | 9.3 (5.1 – 13.9)$^c$ |
| **BCR-ABL1 transcript level after 12 months** | 42 | 0.091 ± 0.096 |
| **Median (range)** | 0.07 (0.04 – 0.67) |

*P-value < 0.001 is highly significant; a: significant difference from level after 3 months; b: significant difference from level after 12 months; c: no significant difference between levels at 3- and 12-months using Wilcoxon test.

### Table 4

|                  | CD26 baseline | CD26 after 3 months | CD26 after 12 months |
|------------------|---------------|---------------------|----------------------|
|                  | r  | $P$  | r  | $P$  | r  | $P$  |
| **Age**          | –0.02 | 0.89 | –0.08 | 0.603 | –0.26 | 0.101 |
| **Spleen size**  | 0.05 | 0.73 | 0.05 | 0.76 | –0.19 | 0.22 |
| **WBCs**         | 0.402 | 0.03$^*$ | 0.514 | 0.001$^*$ | 0.461 | 0.007$^*$ |
| **Platelets**    | –0.327 | 0.03$^*$ | –0.606 | <0.001$^*$ | –0.513 | 0.004$^*$ |
| **HB**           | –0.19 | 0.18 | –0.08 | 0.56 | –0.17 | 0.28 |
| **PB blast**     | –0.14 | 0.35 | –0.24 | 0.54 | –0.19 | 0.87 |
| **BCR-ABL1**     | 0.39 | 0.006$^*$ | 0.318 | 0.03$^*$ | –0.13 | 0.87 |

r: correlation coefficient; **: $P$-value < 0.001 is highly significant; *: $P$-value < 0.05 is significant.
significant correlation between the Sokal index and CD26⁺ LSCs after 12 months of treatment but no significant correlation between the Sokal index and BCR-ABL transcript levels. This result is supported by a previous study that concluded that there was no difference in the BCR-ABL1 transcript levels between Sokal prognostic score risk groups in CP-CML patients treated with imatinib [22].

It is still difficult to decide when to discontinue TKI treatment after DMR in CP-CML patients. It has been demonstrated that in a substantial number of patients who have achieved a stable DMR that TKI treatment can be safely discontinued without loss of response. So, DMR may be regarded as a feasible treatment goal in many CML patients [23]. Some authors suggested the exclusion of the presence of PB CD26⁺ LSCs before TKI discontinuation as it has been described as a specific marker of CML LSCs and is decreased during a successful treatment with TKIs but others denied that as it is still present despite a successful response [11]. Annunziata et al. [24] reported that in clinical practice, TKI withdrawal should be based on expert recommendations and formal guidelines. Treatment discontinuation may be considered in patients with durable DMR with the goal of achieving TFR [25]. However, treatment can be successfully discontinued in a minority of patients depending on whether the durations of both treatment and DMR are long enough to make TFR a feasible option [26]. Other authors recommended BM examination for LSC before the discontinuation of TKI treatment [27].

7. Conclusion

We can conclude that CD26⁺ LSCs are detected in all CP-CML patients at diagnosis and their numbers significantly decreased with TKI treatment though they are still detected in PB in some patients despite the negative BCR-ABL1 after 12 months of treatment, which suggests their usefulness as diagnostic and prognostic markers in these patients.

Funding

None
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