

EXPRESSION OF KI-67 AND CD34 ON BLOOD AND BONE MARROW CELLS OF CML PATIENTS WITH DIFFERENT RESPONSE TO IMATINIB AND NILOTINIB THERAPY

T. Perekhrestenko^{1,*}, U. Melnyk², N. Goryainova¹, I. Diagil³

¹State Institution “Institute of Hematology and Transfusiology of the National Academy of Medical Sciences of Ukraine”, Kyiv 04060, Ukraine

²Kyiv City Clinical Hospital No.9, Kyiv 04112, Ukraine

³State Institution “National Scientific Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine”, Kyiv 004050, Ukraine

Aim: To assess the expression of Ki-67 protein and CD34 antigen on peripheral blood (PB) and bone marrow (BM) cells in chronic myelogenous leukemia (CML) patients with different response to tyrosine kinase inhibitors (TKI) imatinib (IM) and nilotinib (NI) therapy. **Patients and Methods:** BM aspirate and PB samples from 41 CML patients treated with IM and NI were studied by cytogenetic, molecular genetic, and flow cytometry methods. According to the response to TKIs, the patients were distributed into the optimal response, warning, and treatment failure groups. **Results:** The patients with optimal response to TKI therapy showed the lowest levels of Ki-67 expression in PB and BM compared with the patients from warning and failure treatment groups, however, Ki-67 expression was close to the reference values in PB ($0.7 \pm 0.3\%$), only in NI-treated patients. The highest expression of Ki-67 in PB was observed in patients from treatment failure groups. In PB of patients who received NI and did not achieve optimal response, CD34⁺ cell count increased by almost 4 times compared with that in the optimal response group. The results indicated that CD34⁺ cell pool expanded in patients with poor response to both IM and NI. In patients with optimal response to NI therapy, CD34⁺ cell counts in PB were within the reference range and did not exceed 0.5%. Similar results were observed for Ki-67 and CD34⁺ in BM hematopoietic cells. **Conclusions:** Ki-67 expression and CD34⁺ cell count in PB and BM of CML patients increased with the acquisition of clonal resistance to IM and NI. NI provides a deeper molecular response compared with IM.

Key Words: chronic myeloid leukemia, imatinib, nilotinib, CD34⁺ cells, Ki-67.

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Chronic myeloid leukemia (CML), a disease caused by genetic abnormality in the multipotent hematopoietic stem cell, accounts for about 14% of all leukemias and occurs at a frequency of 1–1.5 cases per 100,000 adults. The incidence of CML in Ukraine is about 500–600 cases per year. Over 8,000 new cases of CML are diagnosed annually in the United States [1]. BCR-ABL1 is a fusion protein kinase formed by reverse translocation between chromosomes 9 and 22, which is obligatory and sufficient for the development of CML [2]. Inhibitors of BCR-ABL1 tyrosine kinase (TKI) revolutionized the treatment of CML at the beginning of the XXI century. As a result, the prevalence of CML is growing, as patients on TKIs live with what is more and more viewed as a chronic ailment rather than a potentially lethal disease [3]. It is estimated that over 25% of CML patients will switch TKIs at least once during their lifetime due to TKI intolerance or resistance [4]. The use of second- and third-generation TKIs does not guarantee overcoming the resistance. Although it is known that second-generation TKI drugs, such as nilotinib (NI), which is commonly used in Ukraine with failure of imatinib (IM) therapy, as well as bosu-

tinib and dasatinib, allow deeper molecular response than IM. The most widely studied mechanism of CML resistance to TKI are mutations in the kinase domain of BCR-ABL1, but they cannot explain about 20–40% of resistant cases. Activation of alternative BCR-ABL1-independent survival pathways has been mechanistically implicated in these cases, and may also explain the phenomenon of persistence in responding patients who fail to clear minimal residual disease or experience recurrence upon discontinuation of the therapy despite achieving deep molecular response (DMR, BCR-ABL1 $\leq 0.01\%$ on the international scale) [5].

BCR-ABL gene expresses a membrane-bound tyrosine kinase with constitutive activity that causes uncontrolled cell proliferation. The proliferative capacity of malignant cells might be well assessed by expression of Ki-67 protein [6, 7]. It is also known that the resistance of leukemic clone cells to the TKIs treatment could be caused by the changes in the genome of early CD34⁺ precursor cells that acquire capacity for proliferation with further expansion of CD34⁺ cell pool [8, 9]. Thus, disease prognosis and assessment of CML patients' treatment response can be performed by detecting CD34⁺ cells and monitoring Ki-67 protein in CML patients.

The aim of our study was to assess the expression of Ki-67 protein and CD34 antigen in peripheral blood (PB) and bone marrow (BM) cells in CML patients with different response to IM and NI therapy.

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*Correspondence: E-mail: perekhrestenko.tanya@gmail.com

Abbreviations used: BM – bone marrow; CCR – complete cytogenetic response; CML – chronic myeloid leukemia; IM – imatinib; NI – nilotinib; PB – peripheral blood; TKI – tyrosine kinase inhibitor.

PATIENTS AND METHODS

In the study 41 CML patients at chronic phase of the disease, who received TKI as the first-line therapy, were enrolled. The patients were examined and treated at the State Institution “National Scientific Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine” and “Institute of Hematology and Transfusiology of the National Academy of Medical Sciences of Ukraine”. IM was prescribed to 27 patients and NI was administered to 14 patients. The treatment duration was at least 6 months. The average age of the patients was 48.7 ± 5.2 , and the female/male ratio was 23/18. The study was approved by the local Ethics Review Committee, and all patients gave informed consent for participation in the study.

The response to TKI therapy was evaluated according to the recommendations of National Comprehensive Cancer Network, EuroLeukemiaNet 2013 [10]. The response to TKI therapy and the distribution to the groups of optimal response, warning and failure was evaluated according to the recommendations of National Comprehensive Cancer Network, EuroLeukemiaNet 2013 [10]. The major criterion of the optimal response to TKIs as first-line therapy was the detection level of BCR-ABL transcript ($\leq 0.1\%$ since 12 months of the treatment). In warning group, the cut-off level of BCR-ABL transcript was set up at 0.1–1%, and the failure was assessed when this level was above 1% with the following loss of complete hematological response and complete cytogenetic response (CCR).

To assess the phenotypic characteristics of hematopoietic cells of patients the method of flow cytometry was used. Cytometry study was performed in a flow cytometer FACScan (Becton Dickinson, USA). BM mononuclear cells were stained with monoclonal antibodies: FITC-conjugated mouse anti-human CD34 clone 581 and PE-conjugated mouse anti-human Ki-67 clone B56 (BD Pharmingen, USA). The conjugated antibodies were added directly to PB or BM samples. Following the incubation for 15–30 min at room temperature, the Reagent-10 lysing solution (Becton Dickinson, USA) was added. The cells were washed out in PBS, fixed with 1% paraformaldehyde and analyzed in flow cytometer. The number of cells was determined based on isotype control data.

Upon acquisition, the data were analyzed using the LYSYS-II Ver. 1.1 (Becton Dickinson) and WinMDI 2.8 (Scripps Institute, La Jolla, CA) software. Comparisons of expression of Ki-67, CD34⁺ cells in PB and BM of CML patients with different response to TKI treatment were made by the Mann — Whitney test. The difference was considered significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

The expression of Ki-67 and CD34 was determined in the dynamics of the disease in parallel with the determination of BCR-ABL. The final analysis was performed after 12 months of therapy. According to our data, after 12 month of treatment with IM optimal re-

sponse, warning and treatment failure were observed in 9, 7 and 11 patients, respectively. In patients treated with NI, optimal response, warning and treatment failure were registered in 6, 5 and 3 patients, respectively.

CML patients with optimal response to TKI therapy demonstrated the lowest levels of Ki-67 in PB and BM compared with patients from two other groups. Nevertheless, this index was close to the reference values in PB ($0.7 \pm 0.3\%$) only in patients treated with NI as the first line therapy, whereas in patients receiving IM it was equal to ($2.9 \pm 1.3\%$) (Fig. 1). Therefore, it can be assumed that NI acts more strongly on stem leukemic cells than IM suppressing more actively leukemic cell proliferation.

Comparison between the groups with different responses to TKI treatment showed that the highest expression of Ki-67 in PB was observed in the treatment failure group. In particular, Ki-67 expression was twice higher in the warning group and more than 6 times higher in the treatment failure group compared to the both optimal response groups (treated with IM or NI) ($p < 0.05$). There were no differences in Ki-67 expression between the groups of patients who were assigned to IM and NI and did not achieve optimal response. Similar results were observed in the assessment of Ki-67 expression in BM hematopoietic cells (Fig. 2). Cell count with Ki-67 expression was 2.7 and 3 times higher in the warning and treatment failure groups than in the optimal response groups.

Immunophenotypic quantitative monitoring of CD34⁺ cells in PB and BM was performed in CML patients with different levels of response to IM and NI therapy. Data analysis showed that in PB of patients from treatment failure group, the count of CD34⁺ hematopoietic cells was significantly higher than in patients with optimal response ($p < 0.05$) (see Fig. 1). The expression of CD34 in IM-treated patients in the warning group was 2.4 and 4.3 times higher than in the optimal response group and in the treatment failure group, respectively.

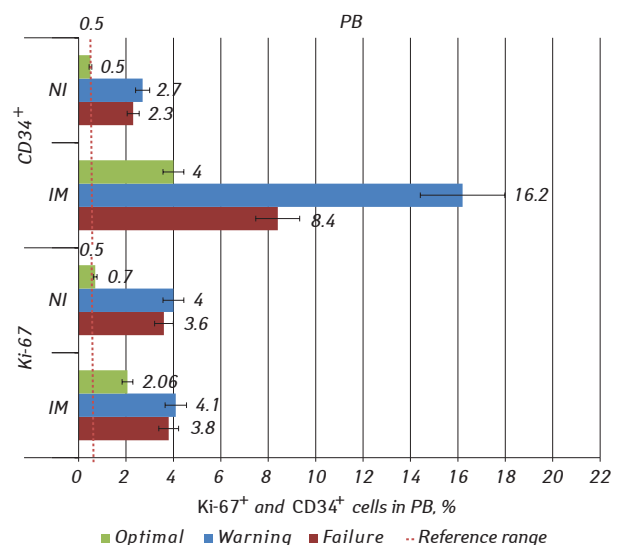


Fig. 1. Cells expressing Ki-67 and CD34 in PB of CML patients with the different response to IM and NI therapy

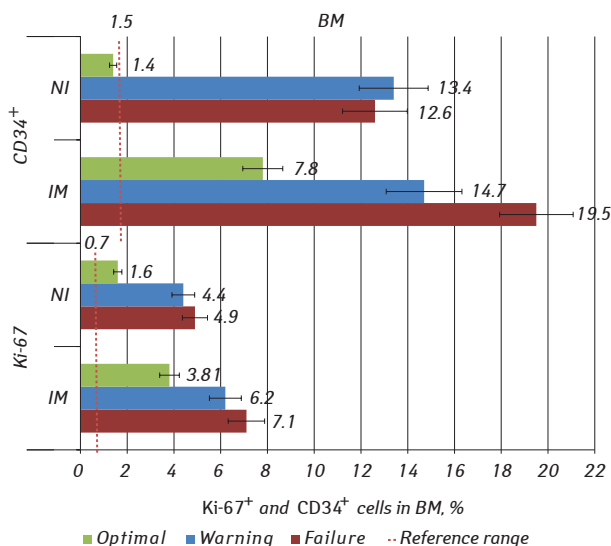


Fig. 2. Cells expressing Ki-67 and CD34 in BM of CML patients with different response to IM and NI therapy

In PB of patients who received NI and did not achieve optimal response, CD34⁺ cell count in PB was almost 4 times higher as compared with the optimal response group. The results suggest that the CD34⁺ cell pool expands in patients with poor response to both IM and NI. It should be noted that in patients with optimal response to NI therapy, CD34⁺ cell count in PB was within the reference values and did not exceed 0.5%. However, in patients who received IM and achieved an optimal response, CD34⁺ cell count did not decrease below 4%. CD34⁺ cell count in BM differed significantly between optimal response groups and treatment failure groups, indicating higher efficiency of NI (see Fig. 2).

CML is the first hematological malignancy with a clear genetic origin (specific chromosomal aberration), and the first one with the developed targeted therapy. However, to date, exact mechanisms of CML progression remain controversial. It is understood that BCR-ABL-independent resistance mechanisms are also capable of protecting leukemic stem cells from the TKI action [11].

In our study, an individual analysis was conducted to evaluate the potential factors for CML progression based on changes in the proliferative activity of leukemic cells by Ki-67 and CD34 expression. As noted above, the main pathognomonic feature of CML is the formation of the chimeric *BCR-ABL* gene, the protein product of which has a significant tyrosine kinase activity, stimulating a large number of intracellular signaling pathways and inhibiting the action of proliferation regulators [2, 3]. As a result, increased proliferation and survival of tumor cells is acquired. One of the well-known markers of cell proliferative activity is the intracellular Ki-67 protein, which is expressed in dividing cells and is absent in the G₀ phase of the cell cycle. The expression of Ki-67 antigen is known to be closely related to biologically aggressive tumor behavior [4].

The identification of cells expressing CD34 in CML patients is necessary not only for clarifying the nature of the cells in the tumor clone, but also for predicting

the course of the disease and for the evaluation of the effectiveness of therapy. Some researchers believe that mutations in the genome of CD34-positive early BM precursor cells are the cause of resistance. Such cells, regardless of the presence of the drug, acquire the ability to actively proliferate and differentiate, and this leads to repeated expansion of the pool of CD34⁺ cells [12].

In this study, we have demonstrated that the functional properties of hematopoietic stem cells and hematopoietic progenitor cells, in particular their proliferative potential may be altered during CML progression. The analysis of the data showed that the count of Ki-67 and CD34⁺ cells in PB and BM of CML patients increases along with the acquisition of clonal resistance to IM and NI. The therapy with NI seems to provide deeper molecular response due to its ability to inhibit the proliferative potential of leukemic stem cells. To date, the inability to achieve a major molecular response (MMR, BCR-ABL1 ≤ 0.1%) in patients with a CCR is debatable because of the unresolved issue of attribution of such clinical cases to therapy failure. Similarly, the interpretation of the confirmed loss of MMR while maintaining CCR needs clarification. Observation of such patients demonstrates that most of them lose CCR over time [13]. In our opinion, the study of the proliferative potential of hematopoietic cells might be used as a prognostic marker of the CCR and MMR loss. We believe that each clinical case requires an individual approach. Careful observation of this group of patients and early switching to other TKIs will prevent the disease progression.

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