EUROPEAN JOURNAL OF CANCER 48 (2012) 233–236

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Quantitative monitoring of BCR/ABL1 mutants for surveillance of subclone-evolution, -expansion, and -depletion in chronic myeloid leukaemia

S. Preuner [a], G. Mitterbauer [b], C. Mannhalter [b], S. Herndlhofer [c], W.R. Sperr [c,d], P. Valent [c,d], T. Lion [a,*]

[a] Children's Cancer Research Institute (CCRI), Vienna, Austria
[b] Institute of Medical Chemistry, Vienna, Austria
[c] Division of Hematology, Medical University Vienna, Austria
[d] Ludwig Boltzmann Cluster Oncology, Vienna, Austria

ARTICLE INFO

Article history:
Available online 28 September 2011

Keywords:
CML
TKD
Mutation
LD-PCR

ABSTRACT

Background: In chronic myeloid leukaemia (CML), clonal evolution with resistance to tyrosine kinase inhibitors (TKIs) is often triggered by BCR/ABL1 mutations. However, in the context of the complex pro-oncogenic signalling networks which ultimately lead to clonal expansion and disease progression, the exact contribution of BCR/ABL1 mutants remains uncertain. Recent data indicate that detection of BCR/ABL1 mutant subclones does not permit prediction of their expansion dynamics and their potential to become drivers of resistant disease.
Methods: To determine the patterns of clonal evolution and the distinct proliferation kinetics of individual BCR/ABL1 mutants during treatment, we employed ligase-dependent polymerase chain reaction (LD-PCR) analysis for quantitative surveillance of CML subclones with various tyrosine kinase domain (TKD) mutations including M244V, L248V, G250E, E255K, T315I, F317L-A/G, M351T and F359V.
Findings: Inadequate treatment responses were observed in 27 of 100 patients investigated and 16 were found to bear one or more BCR/ABL1 TKD mutations in separate subclones. Rapid subclone expansion upon onset or switch of TKI treatment was common and sometimes preceded corresponding changes in BCR/ABL1 transcript levels. Mutant subclones were found to respond differentially and sometimes unexpectedly to various treatment modalities. Decline and persistent depletion of specific mutation-bearing subclones in response to treatment could be documented by LD-PCR surveillance.
Interpretation: The observations show that quantitative monitoring of mutant BCR/ABL1 subclones by LD-PCR is a powerful tool for detection of clonal evolution, subclone-expansion and subclone-depletion and can contribute to optimised management of patients with CML.

1. Introduction

Point mutations within the BCR/ABL1 tyrosine kinase domain (TKD) are currently regarded as the most important mechanism of resistance to tyrosine kinase inhibitors. Although more than 100 different mutations have been described to date, a subset of 15 common mutations are observed in a great majority (>85%) of instances.[1–6] Recent data indicate that the

* *Corresponding author:* Address: Children's Cancer Research Institute (CCRI), Zimmermannplatz 10, A-1090 Vienna, Austria. Tel.: +43 1 40470 4890.
E-mail address: thomas.lion@ccri.at (T. Lion).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2011.08.015

identification of TKD mutations in patients treated with imatinib may be the first sign of impending onset of resistant disease, preceding other indicators of impaired response by several months.[7,8] Moreover, patients harbouring BCR/ABL1 TKD point mutations were reported to have inferior progression-free survival on imatinib to patients without point mutations.[9,10] By contrast, detection of mutant subclones, especially by highly sensitive technical approaches, did not necessarily imply impending onset of clinically resistant disease,[11] and identification of mutations before therapy did not predict insensitivity to imatinib.[12,13] Certain mutations are frequently associated with the onset of resistant disease ('driver mutations'), but a number of other mutations ('bystander' or 'passenger' mutations) may be biologically neutral with regard to tyrosine kinase inhibitor (TKI) resistance.[14,15] However, even mutant subclones commonly associated with resistance to TKI treatment have been described to disappear spontaneously below the limit of detection and to remain undetectable.[13,16] Although mutational screening is most commonly performed by direct sequencing of the entire BCR/ABL1 TKD amplified by polymerase chain reaction (PCR), which does not reveal the presence of mutant subclones below the level of 10–20% of the Ph-positive cell pool, the detection of mutations may still be difficult to interpret with regard to clinical relevance and therapeutic consequences.

We hypothesised that somewhat more sensitive detection of mutant subclones, and close monitoring of their proliferation kinetics could permit *in vivo* assessment of their biological behaviour and indicate resistance to ongoing treatment. To address this question, we have employed a quantitative PCR-based technique (ligase-dependent (LD)-PCR) permitting the detection of individual mutations with a detection limit of $\geqslant$1%, and facilitating highly reproducible and accurate assessment of changes in the size of mutant subclones exceeding ±5%.[17] Prospectively collected serial blood specimens derived from CML patients displaying a variety of mutations were investigated, and the results provide intriguing new insights into the evolution of point-mutated subclones during the course of therapy. The findings presented reveal a way to improved surveillance and treatment of CML patients carrying BCR/ABL1 TKD point mutations.

2. Patients and methods

2.1. Clinical specimens

During the study period from 01/2003 to 12/2010, 100 patients diagnosed with CML were treated in the Department of Hematology at the Medical University Vienna (MUV), Austria. Suboptimal responses to TKI therapy according to ELN criteria and/or rise of BCR/ABL1^{IS} >1% triggered mutational analysis in 27 instances, and point mutations in the BCR/ABL1 TKD were identified by direct sequencing in 16 patients. Serial peripheral blood specimens from nine individuals who mostly displayed multiple mutant subclones during the course of treatment were subjected to detailed quantitative analysis of clonal evolution upon approval of the institutional ethics committee. For the investigation of mutant subclone kinetics, residual archived cDNA was employed, which had been prepared for expression analysis of BCR/ABL1 transcripts and for mutational screening with informed consent of the patients.

2.2. Molecular testing

Quantification of BCR/ABL1 transcripts was performed by real-time PCR according to the International Scale in our ELN-certified laboratory. Screening for mutations in the BCR/ABL1 TKD was carried out using bidirectional sequencing of amplicons generated by semi-nested PCR amplification of the entire kinase domain. Prior to LD-PCR analysis, the cDNA concentration was determined by the Nanodrop 1000 instrument, and 5 μg cDNA was used as template for amplification of the BCR/ABL1 TKD by semi-nested PCR.[18] Sensitive detection of mutant subclones and quantitative monitoring of their relative size within the total BCR/ABL1 positive clone during treatment were performed by LD-PCR in triplicate analyses, as described in detail elsewhere.[17] The percentage of point-mutated cells was expressed in relation to non-mutated cells within the BCR/ABL1-positive CML clone.

3. Results and discussion

In the cohort of CML patients studied, quantitative monitoring of subclones carrying a number of common point mutations in the BCR/ABL1 TKD including M244V, L248V, G250E, E255K, T315I, F317L-A/G, M351T, and F359V was performed by LD-PCR.[17] During a median observation period of 48 months (range 10–109), an average of 18 consecutive peripheral blood specimens (range 5–27) were analysed in each patient. In the select series of CML patients presented, a number of important principles were demonstrated:

Different mutant subclones appeared concomitantly or sequentially in individual patients during treatment, as illustrated in two exemplary cases (Figs. 1 and 2). Changes in the total BCR/ABL1 transcripts assessed by RQ-PCR mostly occurred in parallel to corresponding kinetics of mutant subclones. However, increases in the size of mutant subclones in relation to the entire CML cell pool were also observed when BCR/ABL1 transcripts were still decreasing (Fig. 1), and documentation of expanding mutant subclones occasionally preceded the rise in BCR/ABL1 transcripts by several weeks (mean 9 weeks; range 2–19 weeks) (Figs. 1 and 2). The expansion kinetics was unpredictable and the time span until mutant leukaemic cells became the dominant BCR/ABL1-positive clone was highly variable, ranging between 1 month and more than 2 years after first detection by LD-PCR. Particularly rapid achievement of subclone dominance, within 1 or 2 months, was observed in two patients with leukaemic cells displaying the highly resistant T315I mutation. Conversely, disappearance of a T315I-positive mutant subclone below the detection limit of LD-PCR was also observed in a patient during treatment with hydroxyurea, indicating that even the detection of mutations known to confer a high level of resistance does not necessarily herald imminent refractory disease.

Our study revealed expected findings, such as rapid appearance and expansion of subclones exhibiting the G250E or M244V mutation during treatment with imatinib, the E255K mutation on nilotinib or the F317L-G mutation on dasatinib,[19]

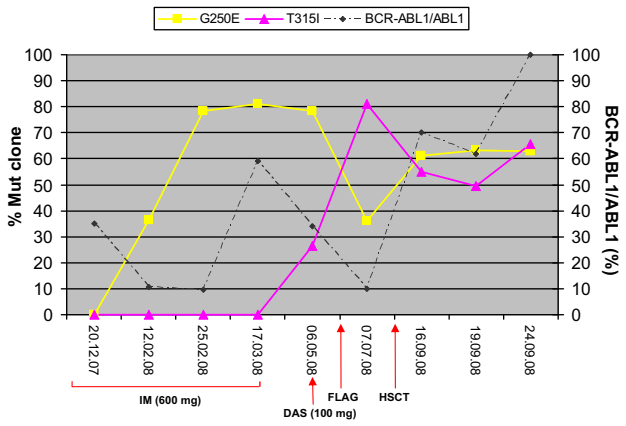

Fig. 1 – Monitoring of molecular response and kinetics of different mutant subclones: exemplary case 1. The proliferation dynamics of two mutant subclones over a period of 9 months is plotted against the BCR/ABL1 transcript levels (black dotted line). The time points of investigation and the treatment modalities are displayed on the X-axis, while the Y-axis shows the BCR/ABL1 expression according to the international scale (IS) and the relative sizes of mutant subclones within the Ph+ cell pool. A G250E-positive subclone appeared and increased in size during the first months of treatment with imatinib (IM) in the presence of decreasing BCR/ABL1 transcripts, and remained at a high level during the entire observation period. An additional subclone carrying the T315I mutation appeared at a later point of treatment, and showed a rapid increase in size while the BCR/ABL-1 transcripts and the G250E mutant cells were decreasing. Expansion of the T315I-positive subclone preceded the increase in the BCR/ABL1 transcripts by about 4 months. Following consecutive treatment attempts with dasatinib (DAS), chemotherapy with FLAG [Fludarabine, Cytosine Arabinoside, Granulocyte colony stimulating factor (GCSF)] and allogeneic haematopoietic stem cell transplantation (HSCT), the patient died in blast crisis about 2 months later.

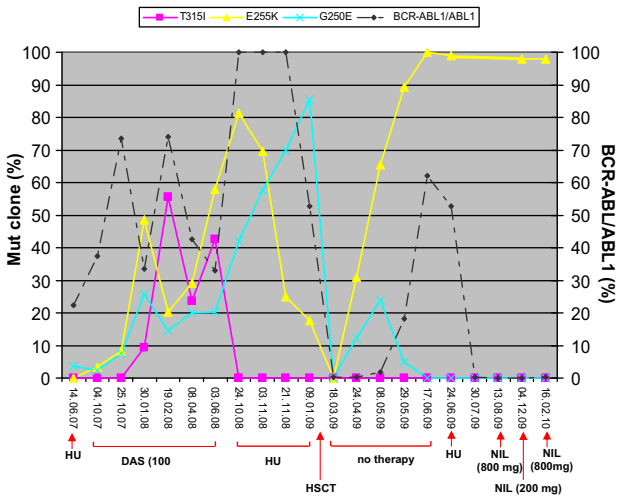

Fig. 2 – Monitoring of molecular response and kinetics of different mutant subclones: exemplary case 2. The proliferation dynamics of three mutant subclones over a period of 2.8 years is plotted against the BCR/ABL1 transcript levels (black dotted line). Following an initial treatment course with hydroxyurea (HU), a T315I-positive subclone appeared after about 4 months of subsequent treatment with dasatinib (DAS), reached a maximum size of 50–60% of the total BCR-ABL1 positive cell pool and, upon onset of treatment with hydroxyurea, disappeared below the detection limit of the LD-PCR technique during the remaining course of disease. A G250E-positive subclone increased steadily during the first 15 months of treatment with hydroxyurea and dasatinib to a maximum of 85%, and ultimately disappeared below the detection limit of the assay after haematopoietic stem cell transplantation (HSCT). Another subclone with the p-loop mutation E255K increased during the first 12 months of treatment to a maximum of >80%, subsequently decreased below the LD-PCR detection limit on hydroxyurea, and expanded rapidly again after HSCT, preceding the rise in BCR/ABL1 transcripts by a few weeks. It has remained the dominant (apparently resistant) subclone during the subsequent treatment with hydroxyurea and nilotinib (NIL), while BCR/ABL1 transcripts decreased on nilotinib to levels of 0.053% according to the International Scale. Following a rise of the BCR/ABL1 levels to 0.25% (not discernable on the scale presented), which corresponded to loss of major molecular response (MMR), the patient died in October, 2010, in chronic phase CML.

which mostly decreased in size upon administration of another TKI, and ultimately disappeared below the detection level of LD-PCR. However, rather unexpected observations were also made, including the documentation of a rapidly proliferating subclone carrying the L248V mutation on nilotinib (not shown), which was anticipated to be effective in this instance based on data obtained *in vitro*. Such findings indicate that point mutations in the BCR/ABL1 TKD with expected sensitivity to certain TKIs may be unresponsive *in vivo* or may merely represent markers of subclones displaying independent mechanisms of resistance. It is therefore important to emphasise that the potency of individual TKIs observed *in vitro* may be insufficient to predict clinical responses and to guide optimal choice of treatment.

Our data demonstrate that the quantitative LD-PCR approach used permits early detection of relevant mutant subclones and accurate surveillance of subclone evolution. Furthermore, the technique facilitates monitoring of the response of individual subclones to treatment. The rapid appearance of mutant cells following changes of treatment supports the notion that CML is composed of various subclones, many of which carry point mutations in the TKD, indicating a non-linear, branching clonal architecture of the disease.[20–22]

Quantitative monitoring during treatment permits reliable assessment of mutant subclone evolution, including both expansion and depletion. Surveillance of mutant subclones by LD-PCR therefore provides information on their actual responsiveness to therapy and the imminent onset of resistant disease. Implementation of quantitative approaches for the monitoring of mutant subclones in the surveillance of CML patients can improve our current options for timely treatment decisions, and may help optimising disease management in patients displaying point mutations in the BCR/

ABL1 TKD or other sites of potential relevance. In view of the emerging knowledge on the multiclonal architecture of leukaemias,[21,22] the intriguing insights derived from the monitoring of individual subclones in CML could serve as a paradigm for different types haematologic malignancies.

Conflict of interest statement

T.L. is consultant at Novartis and Bristol-Myers Squibb (BMS), received honorarium from Novartis and BMS, and research grant from Novartis. P.V. is consultant at Novartis, received honorarium from Novartis, BMS and Pfizer, and research grant from BMS. The remaining authors have no conflict of interest.

REFERENCES

1. Jabbour E, Kantarjian H, Jones D, et al. Frequency and clinical significance of BCR-ABL mutations in patients with chronic myeloid leukemia treated with imatinib mesylate. *Leukemia* 2006;**20**:1767–73.
2. Soverini S, Colarossi S, Gnani A, et al. Contribution of ABL kinase domain mutations to imatinib resistance in different subsets of Philadelphia-positive patients: by the GIMEMA Working Party on Chronic Myeloid Leukemia. *Clin Cancer Res* 2006;**12**:7374–9.
3. Nicolini FE, Corm S, Le QH, et al. Mutation status and clinical outcome of 89 imatinib mesylate-resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (Fi(phi)-LMC GROUP). *Leukemia* 2006;**20**:1061–6.
4. Branford S. Chronic myeloid leukemia: molecular monitoring in clinical practice. *Hematol Am Soc Hematol Edu Program* 2007:376–83.
5. Apperley JF. Part I: Mechanisms of resistance to imatinib in chronic myeloid leukaemia. *Lancet Oncol* 2007;**8**:1018–29.
6. Branford S, Melo JV, Hughes TP. Selecting optimal second-line tyrosine kinase inhibitor therapy for chronic myeloid leukemia patients after imatinib failure: does the BCR-ABL mutation status really matter? *Blood* 2009;**114**:5426–35.
7. de Lavallade H, Apperley JF, Khorashad JS, et al. Imatinib for newly diagnosed patients with chronic myeloid leukemia: incidence of sustained responses in an intention-to-treat analysis. *J Clin Oncol* 2008;**26**:3358–63.
8. Khorashad JS, de Lavallade H, Apperley JF, et al. Finding of kinase domain mutations in patients with chronic phase chronic myeloid leukemia responding to imatinib may identify those at high risk of disease progression. *J Clin Oncol* 2008;**26**:4806–13.
9. Press RD, Willis SG, Laudadio J, Mauro MJ, Deininger MW. Determining the rise in BCR-ABL RNA that optimally predicts a kinase domain mutation in patients with chronic myeloid leukemia on imatinib. *Blood* 2009;**114**:2598–605.
10. Sharma P, Mohanty S, Kochupillai V, Kumar L. Mutations in ABL kinase domain are associated with inferior progression-free survival. *Leuk Lymphoma* 2010;**51**:1072–8.
11. Willis SG, Lange T, Demehri S, et al. High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy. *Blood* 2005;**106**:2128–37.
12. Jabbour E, Branford S, Saglio G, et al. Practical advice for determining the role of BCR-ABL mutations in guiding tyrosine kinase inhibitor therapy in patients with chronic myeloid leukemia. *Cancer* 2011;**117**:1800–11.
13. Khorashad JS, Anand M, Marin D, et al. The presence of a BCR-ABL mutant allele in CML does not always explain clinical resistance to imatinib. *Leukemia* 2006;**20**:658–63.
14. Sherbenou DW, Hantschel O, Kaupe I, et al. BCR-ABL SH3-SH2 domain mutations in chronic myeloid leukemia patients on imatinib. *Blood* 2010;**116**:3278–85.
15. Branford S, Hughes TP. Practical considerations for monitoring patients with chronic myeloid leukemia. *Semin Hematol* 2010;**47**:327–34.
16. Khorashad JS, Milojkovic D, Mehta P, et al. In vivo kinetics of kinase domain mutations in CML patients treated with dasatinib after failing imatinib. *Blood* 2008;**111**:2378–81.
17. Preuner S, Denk D, Frommlet F, Nesslboeck M, Lion T. Quantitative monitoring of cell clones carrying point mutations in the BCR-ABL tyrosine kinase domain by ligation-dependent polymerase chain reaction (LD-PCR). *Leukemia* 2008;**22**:1956–61.
18. Branford S, Hughes T. Detection of BCR-ABL mutations and resistance to imatinib mesylate. *Methods Mol Med* 2006;**125**:93–106.
19. Soverini S, Gnani A, Colarossi S, et al. Philadelphia-positive patients who already harbor imatinib-resistant Bcr-Abl kinase domain mutations have a higher likelihood of developing additional mutations associated with resistance to second- or third-line tyrosine kinase inhibitors. *Blood* 2009;**114**:2168–71.
20. Valent P. Targeting of leukemia-initiating cells to develop curative drug therapies: straightforward but nontrivial concept. *Curr Cancer Drug Targets* 2010;**11**:56–71.
21. Greaves M. Cancer stem cells: back to Darwin? *Semin Cancer Biol* 2010;**20**:65–70.
22. Notta F, Mullighan CG, Wang JC, et al. Evolution of human BCR-ABL1 lymphoblastic leukaemia-initiating cells. *Nature* 2011;**469**:362–7.