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REVIEW ARTICLE

Molecular monitoring of chronic myeloid leukemia: principles and interlaboratory standardization

Nicholas C. P. Cross · Andreas Hochhaus · Martin C. Müller

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Abstract Serial quantification of *BCR-ABL1* messenger RNA (mRNA) is an important therapeutic indicator for patients with chronic myeloid leukemia, but historically, there has been substantial variation in results reported by different laboratories. To help improve the comparability of results, an international scale (IS) for *BCR-ABL1* was proposed which is being implemented by testing laboratories worldwide. This is being achieved most commonly by the derivation of laboratory-specific conversion factors, but increasingly by the use of kits or reagents that are calibrated to the first World Health Organization International Genetic Reference Panel for quantitation of *BCR-ABL1* mRNA. Recent attention has focused on the need to define and validate levels of deeper molecular response (MR) within the context of the IS. While there has been substantial progress in the alignment of results, *BCR-ABL1* measurement is technically challenging and standardization is an ongoing process.

Keywords Chronic myeloid leukemia · BCR-ABL1 mRNA · BCR-ABL1 measurement

N. C. P. Cross
Faculty of Medicine, University of Southampton, Southampton, UK

N. C. P. Cross (✉)
Wessex Regional Genetics Laboratory, Salisbury District Hospital, Salisbury SP2 8BJ, UK
e-mail: ncpc@soton.ac.uk

A. Hochhaus
Abteilung Hämatologie/Onkologie, Klinik für Innere Medizin II, Universitätsklinikum Jena, Jena, Germany

M. C. Müller
III. Medizinische Klinik, Universitätsmedizin Mannheim, Mannheim, Germany

Background

Studies to explore the possibility of molecular monitoring of chronic myeloid leukemia (CML) patients by reverse transcription polymerase chain reaction (RT-PCR) were initiated more than 25 years ago, when the principal clinical challenge was to develop a methodology to detect early relapse after bone marrow transplantation (BMT). The first studies were qualitative, using nested RT-PCR and standard agarose gel electrophoresis to determine whether *BCR-ABL1* messenger RNA (mRNA) was detectable or undetectable in patient samples, with a control for adequate complementary DNA (cDNA) quality being provided by single-step amplification of a housekeeping control gene. Although some of these studies were able to identify groups of patients that were more or less likely to relapse, the predictive value for individual cases was very limited [1–5] and thus there was a need to develop quantitative RT-PCR approaches that might be able to give an indication of the level of disease in specimens that tested positive for *BCR-ABL1* and the kinetics of the malignant clone over time. However, marked differences were apparent between centers and early attempts at standardization focused mainly on the need to eliminate false-positive results arising from contamination of amplification reactions with previously amplified products [6].

Initial quantitative procedures were based on the use of competitive PCR, which relies on the addition of known numbers of molecules of a competitor plasmid to a series of amplification reactions, with the number of *BCR-ABL1* targets in the sample being estimated by determining the point at which the competitor and *BCR-ABL1* amplicons are of equivalent fluorescent intensity on an agarose gel. Using competitive PCR, it was shown that rising *BCR-ABL1* levels on sequential analysis predicted relapse after BMT and provided prognostically useful information for patients in complete cytogenetic remission (CCyR) on interferon alpha [7–11].

Competitive PCR was thus effective but extremely labor intensive and was only performed on a research basis in a small number of transplant centers. The development and subsequent commercialization of reverse transcription real-time quantitative PCR (RT-qPCR) in the late 1990s along with the introduction of highly effective targeted therapy for CML provided the means and the need for widespread adoption of molecular monitoring. However, there was no standard approach as to how essential elements of the assay should be performed and different methodological variants began to proliferate which resulted in highly variable or even incomparable results between centers. The challenge in recent years has been to create the means by which molecular monitoring results can be standardized across testing centers internationally.

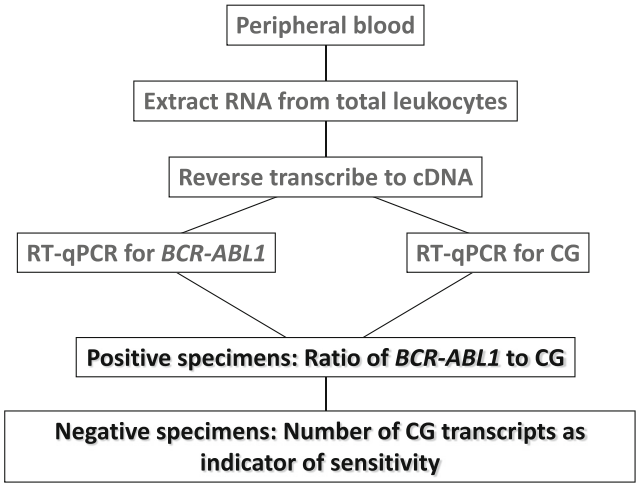

Fig. 1 Schematic outline of *BCR-ABL1* RT-qPCR analysis. Positive specimens are those in which *BCR-ABL1* mRNA is detected, and negative specimens are those in which *BCR-ABL1* mRNA is not detected. *CG* control gene

Measurement of residual disease in the laboratory

To understand the problem of standardization, it is first necessary to understand how the test is typically performed. Anticoagulated peripheral blood or bone marrow samples are received in the testing laboratory, ideally within 24 h of collection. Generally, the peripheral blood is preferred as this is less invasive and results are comparable to bone marrow provided that total leukocytes are recovered by lysis of red cells [12]; in contrast to the analysis of residual disease in acute leukemia, mononuclear cells isolated by density gradients such as Lymphoprep® or Ficoll® should not be used for CML. Leukocytes are lysed in a chaotropic agent that inactivates pervasive RNA-degrading enzymes, and RNA is extracted and reversely transcribed to cDNA, typically using random hexamer primers (Fig. 1). Differences in the amount of RNA extracted, the integrity of that RNA, and the efficiency with which it is reversely transcribed may vary widely between samples, even in established laboratories. This means that the sensitivity with which *BCR-ABL1* can be detected or excluded is also highly variable. It is generally agreed that the best way to take this variation into account is to relate the number of copies of *BCR-ABL1* to those of a housekeeping control gene, which serves as an internal control for both the quantity and quality of the cDNA for each sample [13].

Consequently, two measurements are made by RT-qPCR for all samples: an estimate of the number of *BCR-ABL1* transcripts and an estimate of the number of transcripts of a control gene (CG). Different laboratories use various processes to derive these estimates; for example, some measure *BCR-ABL1* and the CG singly, in duplicate or in triplicate from an identical cDNA specimen; others make a single measurement from independent cDNA preparations. In addition, different criteria are used to define whether a result is considered detectable or undetectable based on replicate results and technical parameters.

Results for specimens that are considered to test positive for *BCR-ABL1* are expressed as the ratio of *BCR-ABL1* transcript numbers divided by the number of control gene transcripts in the same volume of cDNA. For samples that test negative for *BCR-ABL1*, the value of the CG gives an indication of the sensitivity with which residual disease can be excluded for that particular specimen. It is very important that testing laboratories monitor closely the variability of their assay and reject runs that are considered as outliers, for example by regularly measuring high and low standards [14, 15]. Establishment of RT-qPCR requires extensive validation, for example using the methodology described by the Molecular Oncology Resource Committee of the College of American Pathologists [16].

Choice of control gene

One of the critical variables between centers has been the choice of CG. An ideal CG would be expressed uniformly in different cell types regardless of their proliferative status, unaffected by therapeutic regimens, invariant between individuals, and expressed a level similar to *BCR-ABL1*. Unfortunately, such a CG does not appear to exist and no single CG has emerged that is clearly best, although the most widely used is *ABL1*. This is partly a historical accident as the plasmid constructs that were used for competitive PCR quantification could also be used to quantify normal *ABL1* expression [7]. Subsequently, however, the Europe Against Cancer (EAC) group undertook an extensive analysis of candidate CGs and concluded that *ABL1*, beta-2-microglobulin (*B2M*),

and beta-glucuronidase (*GUSB*) were suitable for normalization of RT-qPCR results [13]. The normal *BCR* gene is also widely used as an internal control for CML, based on the rationale that both normal *BCR* and *BCR-ABL1* are driven by the same promoter, and thus, they are likely to be transcribed at similar rates in different cell types [14]. Although some other genes are also used, the great majority of testing laboratories use *ABL1*, *BCR*, *GUSB*, or *B2M* as internal controls. This means that there are at least four units of measurement in its widespread use for the estimation of residual disease in CML: *BCR-ABL1*/total *ABL1* (i.e., *ABL1*+*BCR-ABL1*), *BCR-ABL1*/*BCR*, *BCR-ABL1*/*GUSB*, and *BCR-ABL1*/*B2M*.

Although the use of different control genes is the principal reason, historically, for limited comparability of results between centers, there are also other important factors that are particularly relevant for laboratory-developed tests. Laboratories using the same CG often use different probe/primer combinations, partly as a result of concerns about infringements of intellectual property rights. In addition, laboratories may differ in their approach to the setting of user-defined parameters such as the threshold, what constitutes an acceptable result in terms of the slope of the standard curve, the minimum number of points to construct a standard curve, what cycle threshold (C_t) value is accepted as a positive result, and the reproducibility between duplicate or ideally triplicate replicates. Finally, in order to achieve sensitive detection of residual disease, it is essential to analyze a sufficiently large sample. Clearly, it is impossible to achieve a sensitivity of 1 in 10^5 if only the equivalent of 10^4 cells or fewer are analyzed. Some of these issues have been addressed by the EAC and consensus guidelines published [17, 18]; in addition, many commercially available kits provide detailed guidance for RT-qPCR setup and analysis.

The international scale for *BCR-ABL1* measurement

The International Randomized Study of Interferon and STI571 (IRIS) study demonstrated the dramatic superiority of imatinib over interferon-based regimens. In this trial, RT-qPCR analysis was centralized in three centers that used different laboratory procedures and two different control genes [19]. Large, reproducible differences in median *BCR-ABL1* values at specific time points between the three centers were noted which prompted the need for an urgent alignment of their respective results (Fig. 2). In the absence of any independent reference or calibration materials, an essentially arbitrary decision was made that each center would measure the level of disease in a common set of 30 pretreatment CML patient samples using *BCR* as a control gene and the results would be normalized to this standardized baseline. Reanalysis of the data showed improved comparability of results between the three laboratories, and the standardized baseline was used to normalize all subsequent trial results [19]. Thus, major molecular response (MMR), for example, is defined as a three-log reduction from the IRIS-standardized baseline and not a three-log reduction from a pretreatment material for each individual case.

The scale used in the IRIS trial subsequently formed the basis of the international scale (IS) for *BCR-ABL1* measurement, which was proposed as a means to enable laboratories to produce results that are, in principle, more comparable between centers while continuing to use their existing procedures [20]. Although the samples used for definition of the IRIS-standardized baseline were very limited in quantity and therefore quickly exhausted, excellent traceability to the IRIS measurement was provided by the detailed internal quality control data accrued by the Adelaide laboratory [14]. The IS expresses a detectable disease as a percentage, with 100 %

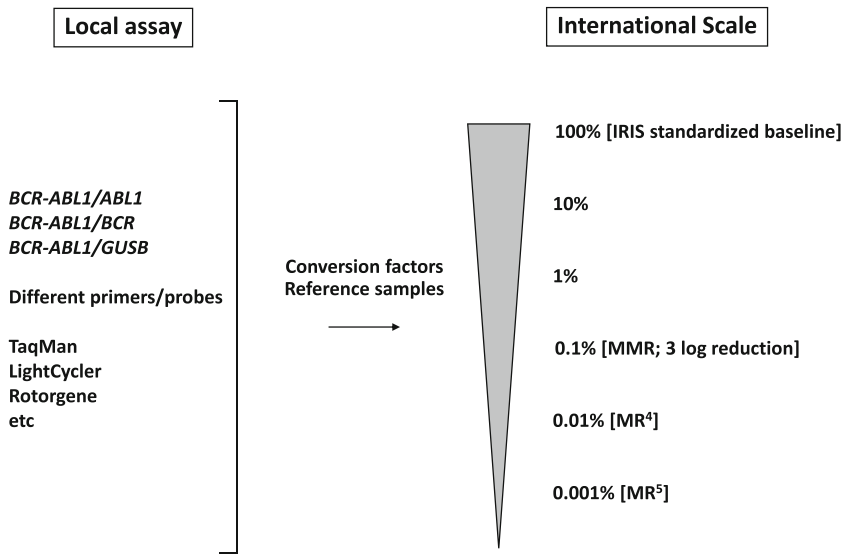

Fig. 2 The international scale for *BCR-ABL1* RT-qPCR measurement. Centers continue to use their established systems for *BCR-ABL1* and convert results to the international scale (IS) using CFs or calibrated reference reagents. *MMR* major molecular response; MR^4 and MR^5 are four- and five-log reductions, respectively, from the IRIS-standardized baseline

BCR-ABL^{IS} defined as the IRIS-standardized baseline and 0.1 % BCR-ABL^{IS} corresponding to MMR. A level of 1 % BCR-ABL^{IS} corresponds roughly to the limit at which Ph-positive metaphases can be detected by standard cytogenetics, and thus, lower levels of disease are consistent with CCyR [21].

The initial focus of the IS was on detectable residual disease and, in particular, whether a patient had or had not achieved defined milestones, for example 10 or 0.1 % BCR-ABL^{IS}. Second-generation TKIs produce faster and deeper responses compared to imatinib, and the need has arisen for robust, standardized, and workable definitions of deep molecular responses [22]. Such definitions are particularly important in the context of ongoing studies that are exploring the possibility of treatment cessation in patients who achieve sustained good responses.

Although the terms "complete molecular response" and "complete molecular remission" have been used in the past, it is difficult to define these terms in any meaningful way. Instead, it has been suggested that definitions of deep response need to be qualified with the level of sensitivity achieved for that sample, particularly for specimens where *BCR-ABL1* is not detected. The following definitions have been proposed [22] and accepted by the European LeukaemiaNet in their 2013 recommendations for the management of CML patients [23]:

- MR^4 (greater than or equal to four-log reduction from IRIS baseline)=either (i) detectable disease ≤0.01 % *BCR-ABL1*^{IS} or (ii) undetectable disease in cDNA with ≥10,000 *ABL1* transcripts*
- MR$^{4.5}$ (≥4.5-log reduction from IRIS baseline)=either (i) detectable disease ≤0.0032 % *BCR-ABL1*^{IS} or (ii) undetectable disease with in cDNA with ≥32,000 *ABL1* transcripts*

*Numbers of *ABL1* transcripts (or equivalent for other CGs) in the same volume of cDNA used to test for *BCR-ABL1*

These definitions depend critically on the ability of testing laboratories to measure absolute numbers of *CG* transcripts in a comparable manner as well as their ability to achieve the requisite sensitivity. In addition, there is a considerable variation in the way in which testing laboratories define undetectable or low-level disease and the European Treatment and Outcome Study (EUTOS) group within the European LeukemiaNet (www.leukemia-net.org) has developed precise laboratory definitions and performance criteria to define deepmolecular response [24].

Implementing the international scale

Although the concept of the IS is very attractive, international implementation has been proven to be challenging. Initially, the only mechanism for laboratories to adopt the IS was to establish a laboratory-specific conversion factor (CF) using a process initiated by the Adelaide laboratory (33). For a testing laboratory to establish a CF, a series of samples (typically 20–30) are exchanged with a reference laboratory that span at least three logs of detectable disease but do not exceed an IS value of roughly 10 %. Samples are analyzed by both centers over a period of 2–3 months typically in order to take into account common intralaboratory variables, e.g., different operators and different batches of reagents. The results for the reference and test laboratories (using the IS and local units, respectively) are compared, and the CF for the testing laboratory is derived by straightforward mathematical calculation. To validate the CF, a further set of samples are exchanged which are again analyzed in a similar manner, i.e., in both centers over a period of time. If the converted values for the test laboratory show a bias of within ±1.2-fold compared to the reference laboratory, then the CF is considered validated and suitable for conversion of the test laboratory results to the IS. Due to the complexity of the process and the need to derive a new CF if elements of the process are changed, it is essential that the RT-qPCR assay and all associated processes are fully optimized in the test laboratory prior to sample exchange. Of 38 test laboratories with which undertook this process (using 19 different methods and 5 different control genes), 22 (58 %) successfully established validate CFs, testifying to the success of the process [25]. The reason that the validation process failed in the remaining test laboratories is unclear but presumably indicates that their assays are nonlinear or unstable over time.

Since it is impossible for a single reference laboratory to standardize all other testing laboratories in the world, the concept of regional or national reference laboratories has been developed, for example in Europe through EUTOS. Following derivation of a CF with Adelaide, the laboratory in Mannheim has performed sample exchanges and derived further CFs with more than 50 testing centers which can then serve in turn as reference centers for their countries or regions [26]. Although this process has worked well, at least for laboratories with stable assays, it is arguably intrinsically flawed as any errors will be propagated along the line. Furthermore, there are other obvious issues, e.g., (i) derivation of CFs is time consuming and expensive; (ii) due to the requirement to involve an established reference laboratory, the process is only open to a limited number of testing laboratories at any given time; (iii) many centers struggle to accrue sufficient numbers of suitable samples; (iv) it is unclear how often CFs need to be revalidated; (v) it is unclear what happens to the 50 % of laboratories who fail to achieve the defined performance criteria; and (vi) it is unclear what constitutes a stable or unstable CF and how testing laboratories should accommodate CFs that change over time.

Development of reference reagents and calibrated kits

While the development of CFs was a major step forward and provided an important proof of principle, it is obvious that this approach is not sustainable in the long term. Ideally, any testing laboratory should be able to access reference standards or use a kit that enables them to convert patient results directly to the IS. The development of standards and kits initially required the development of a process by which these tools could be calibrated to the IS. An important milestone in this process was the establishment in 2010 of the first World Health Organization International Genetic Reference Panel for quantitation of *BCR-ABL1* mRNA [27]. The reference panel comprises four different dilution levels of freeze-dried preparations of K562 cells diluted in HL60 cells that were assigned fixed % *BCR-ABL1*/CG values on the IS following an international calibration process. Due to the scale of molecular monitoring, it was not physically possible to manufacture and validate a sufficiently large quantity of reference material to satisfy worldwide demand and thus the principal function of these primary reagents was limited to the calibration of secondary reference reagents. These secondary reference reagents may be manufactured and calibrated by companies, reference laboratories, or other agencies and made available to testing laboratories either on a commercial basis or as part of specific national or regional standardization initiatives. At the time of writing, a number of different kits, systems, and secondary reagents are available that enable testing laboratories to derive patient results on the IS [28, 29]. Comparative data is, however, very limited at present, and it is not possible to say which of these approaches is best, but in principle, the use of calibrated reagents is likely to replace the use of CFs in the coming years [30].

As indicated above, standardization of deep molecular response requires testing laboratories to be able to estimate absolute numbers of CG transcripts in a comparable manner as an indication of the quality of the sample. Determination of the number of *BCR-ABL1* and CG transcripts is typically performed by using an external plasmid calibrator; however, different calibrators (developed in house or commercially available) are in use worldwide and, until very recently, no common reference material existed to which they could be aligned. In response to this, an internationally accepted certified reference plasmid, ERM-AD623, was developed that includes *BCR-ABL1* and the three most commonly used CGs (*ABL1*, *BCR*, and *GUSB*) [31]. The direct or indirect use of this plasmid helps to improve the accuracy of results prior to conversion as well as the accuracy of CG copy number estimates for samples where *BCR-ABL1* is not detected.

What is achievable by standardization?

The combination of CFs and ERM-AD623 should help testing laboratories to generate more standardized results. However, some lack of agreement between different laboratories using diverse methodologies and control genes will remain. Whether this remaining disagreement is acceptable depends on the effect it has on clinical interpretation. When evaluating the performance characteristics of a method, two factors should be considered: trueness (i.e., the degree of closeness of mean measured quantity value and the true quantity value) and precision (i.e., the degree to which repeated measurements under unchanged conditions show the same results). The trueness of a method can be estimated by comparing the average value obtained from several replicate measurements on a reference material with an established IS value. The precision of a method can be estimated from the 95 % limit of agreement of all the individual measurement results obtained for the reference material. Existing experience with the setup and validation of CFs has shown that an average difference within ±1.2-fold of the established value and 95 % limits of agreement within ±5-fold of the established value were achieved using the best performing methods [25]. This led to an MMR concordance rate of 91 %, a level of agreement which probably represents the maximum that can be achieved using the current RT-qPCR technology. However, this figure of 91 % critically depends on the set of samples that are used and would be expected to be substantially lower if the sample set was restricted to samples that were close to MMR. It is important therefore to consider intrinsic assay variation when assessing the response of a patient against specific milestones such as those recommended by the ELN [23]. In addition, at very low levels of disease, a variation between replicates is inevitably greater than that seen at higher levels due to the fact that small numbers of molecules are being sampled. This should be taken into account when interpreting changes in levels of disease on sequential analysis, e.g., a fourfold increase from 0.002 to 0.008 % IS might be considered as a prompt to perform repeat analysis at the next scheduled visit, whereas an increase from 0.07 to 0.28 % would be considered sufficient for rapid repeat analysis and possibly mutation testing [32].

Next steps

Standardization of molecular monitoring is an ongoing process and critically requires testing laboratories to implement robust internal quality control to monitor assay drift and reproducibility [14]. In principle, if a laboratory can demonstrate assay stability over time, then they only need to derive a CF once, although a new CF will have to be derived either

internally or externally if processes or equipment is changed [25]. Current work is focused on defining deep molecular response in a reproducible manner and increasing assay sensitivity to enable at least $MR^{4.5}$ to be achieved in most samples, a level of detection that is considered important to achieve for treatment-free remission studies. Alternative approaches such as amplification of patient-specific genomic DNA *BCR-ABL1* fusions might provide greater insights into the dynamics of the malignant clone [33–35], and technologies such as digital PCR may prove to be more robust for quantifying low levels of disease [36, 37]. However, it seems likely that RT-qPCR will continue to be the method of choice for monitoring CML patients for the foreseeable future.

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Conflict of interest The authors declare that they have no relevant conflict of interest.

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