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Utility of peripheral blood dual color, double fusion fluorescent in situ hybridization for BCR/ABL fusion to assess cytogenetic remission status in chronic myeloid leukemia

ANDREW P. LANDSTROM, RHETT P. KETTERLING, RYAN A. KNUDSON, & AYALEW TEFFERI

Mayo Clinic College of Medicine, Rochester, MN, USA

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Abstract
The molecular signature of BCR-ABL fusion in chronic myeloid leukemia (CML) provides a unique tool for diagnosis and monitoring of tumor burden during therapy. The gold standard in this regard is conventional bone marrow cytogenetics. Peripheral blood fluorescent in situ hybridization (FISH) offers the possibility of a less invasive, more practical alternative method in terms of both cost and turnaround time. In the present study, we examined 296 paired samples from 65 patients with CML and demonstrate a tight correlation, in quantifying BCR-ABL burden between bone marrow cytogenetics, bone marrow dual color, double fusion (D-FISH), and peripheral blood D-FISH (P < 0.0001 for each). Furthermore, we demonstrate that peripheral blood D-FISH can be used as a surrogate for cytogenetic studies in monitoring cytogenetic remission status.

Keywords: Chronic myeloid leukemia, Philadelphia chromosome, cytogenetics, FISH

Introduction
Chronic myeloid leukemia (CML) is a myeloproliferative disorder that is characterized by the reciprocal translocation of the ABL oncogene and the BCR gene, which is usually characterized by t(9;22)(q34;q11.2) [1–3]. This translocation involves a break within a large, approximately 200 kb, region within the first alternate exon of ABL, as well as the smaller breakpoint cluster region (BCR) of 5.8 kb [4,5]. The derivative chromosome 22, known as the Philadelphia chromosome (Ph), contains the novel BCR/ABL fusion gene which serves as a unique molecular marker for diagnosis, prognostication and monitoring of response to treatment [3,6]. Accordingly, several genetic testing techniques have been developed based on detection of the BCR-ABL and are mainstays in the current management of patients with CML.

Conventional cytogenetics (CC) is the gold standard for the initial detection and subsequent quantification of BCR-ABL through its cytogenetic equivalent; however, karyotypic analysis has several limitations, the primary of which is the need for dividing cells for which 25 analysable metaphases are routinely examined. Further limitations include failure to detect submicroscopic or cryptic chromosomal rearrangements, timeliness, cost and invasiveness to the patient [7–9]. In addition, bone marrow samples for CC might not be obtained in adequate quantity due to associated myelofibrosis or treatment with hypoplasia-inducing myelosuppressive drugs. Finally, CC has suboptimal sensitivity for the assessment of minimal residual disease (MRD) which is estimated to be approximately 5% [10–13].

Fluorescence in situ hybridization (FISH) has become an integral part of the evaluation of CML and utilizes fluorescently tagged DNA probes that bind directly to specific loci within the genome. This technique is primarily applied to non-dividing (interphase) nuclei, thus eliminating the need for viable dividing cells require by CC. FISH can routinely be performed on peripheral blood samples, in addition
to bone marrow, thus potentially sparing patients from a highly invasive and costly procedure [14].

Dual color, double fusion FISH (D-FISH), which utilizes probes that span the common breakpoints in the ABL and BCR gene regions, is a strategy that generates two novel fusion signals: one on the derivative chromosome 9 and one on the derivative chromosome 22 (Ph). The creation of these two fusion signals drives the number of false-positive and false-negative events to <0.6% of cells when 500 nuclei are analysed [9]. In the present study, we analysed data from 65 CML patients to explore the correlation between bone marrow CC, bone marrow D-FISH, and peripheral blood D-FISH to assess the application of peripheral blood D-FISH for obtaining measurements of tumor burden compared to CC.

Materials and methods

Cohort

After approval from the Mayo Foundation Institutional Review Board and in accordance with appropriate federal regulations, electronic medical records were searched for patients presenting to the Mayo Clinic with CML. The diagnosis of CML required the demonstration of a t(9;22)(q34;q11.2) by CC and/or demonstration of BCR/ABL fusion by D-FISH. Patients who had received any CML-directed therapy prior to presentation were excluded. These criteria yielded our study cohort, for whom demographic information including gender, age and status at last follow-up visit were noted.

Conventional cytogenetic and FISH analyses

Methods used for CC and D-FISH have previously been described by Dewald et al. [9,15], and we took advantage of three methodologies of BCR-ABL detection: (i) CC; (ii) D-FISH utilizing bone marrow samples; and (iii) D-FISH utilizing peripheral blood samples. We utilized a commercially available BCR/ABL D-FISH probe set strategy (Vysis, Inc., Des Plaines, Illinois, USA) for which D-FISH stands for dual color, double fusion probe. The BCR/ABL probe set comprises a direct-labelled red (Spectrum Orange, Vysis, Inc.) probe spanning the ABL gene at 9q34 and a direct-labelled green probe (Spectrum Green, Vysis, Inc.) spanning the BCR gene at 22q11.2. Normal interphase nuclei will have a simple 2R2G signal pattern. The 9;22 translocation between ABL and BCR results in a split through both probes, leaving a residual red on the abnormal chromosome 9 and a residual green on the abnormal 22. The translocated red ABL signal then ‘fuses’ with the residual green BCR signal on the abnormal chromosome 22 and the translocated green BCR signal ‘fuses’ with the residual ABL signal on the abnormal chromosome 9. The juxtaposition of the green and red FISH signals produces a yellow fusion color (F). The normal chromosomes 9 and 22 that were not involved in the 9;22 translocation will retain the intact red ABL and green BCR signals, respectively. Thus, abnormal interphase cells with a t(9;22) will display a FISH signal pattern of one red (normal 9), one green (normal 22) and two yellow fusion (abnormal 9 and 22 with BCR/ABL and ABL/BCR fusions). The creation of these two novel fusion signals virtually excludes false-positive nuclei and the normal cut-off is <0.6% of when 500 nuclei are analysed.

These measurements were taken at diagnosis as well as for each follow-up visit for each patient in the cohort. Triplicate and paired samples, which were defined as collections of CC or D-FISH that were obtained within 24 h of each other, were compiled. Specimens taken from bone marrow and blood were not combined for the purposes of the FISH assay, and each sample was analysed as in independent specimen, even if the samples were obtained concurrently from the patient. Because each sample was processed independently, it is unlikely that lymphocytes from the bone marrow would contaminate peripheral blood samples. The progression of CML often leads to dispersion of marrow cells into the blood; however, this forms the basis of FISH peripheral blood detection.

Each paired collection was grouped by CC and bone marrow D-FISH, CC and peripheral blood D-FISH and, finally, bone marrow and peripheral blood D-FISH. For each correlation explored, a linear best-fit model was calculated, as well as the strength via $r^2$ and P-values. All data were analysed using SAS software (SAS Inc., Cary, NC, USA). $P = 0.05$ was considered statistically significant.

Results

Cohort description

The study cohort comprised a pool of 65 subjects who had BCR/ABL-positive CML and had not received prior treatment (34 males and 31 females). Only one patient did not harbor a t(9;22)(q34;q11.2) by CC but a concurrent bone marrow D-FISH revealed BCR-ABL in 92.2% of the cells. The mean ± SD (range) age was 58.9 ± 13.37 (19–84) years. Length of diagnosis after presentation was 3.9 ± 2.429 (range 0–9.42) years, including subjects or which no additional follow-up visits were available. At last follow-up, 55 patients were living whereas ten were deceased.
There were a total of 296 paired samples from the subject pool, 39 of which are triplicate collections, for which CC, bone marrow and peripheral blood D-FISH data were collected within a 24-h period. Including these triplicate collections, there were a total of 176 duplicate collections of CC and bone marrow D-FISH, 70 duplicate CC and peripheral blood D-FISH, and 50 duplicate bone marrow and peripheral blood D-FISH measurements. These duplicate values were taken from 54, 35 and 25 patients, respectively.

**Triplicate laboratory values**

The 39 triplicate patient collections for detection of BCR-ABL are listed in Table I. Collections 1–7 are triplicate collections from subjects at diagnosis and prior to treatment, while collections 8–39 were taken from subject follow-up visits after treatment. In all collections, each of the values for CC, bone marrow D-FISH and peripheral blood D-FISH were in agreement. Any CC value that indicates partial cytogenetic remission (Ph-positive tumor burden of <33%), or complete cytogenetic remission (no Ph detection in all metaphases) demonstrated corresponding D-FISH values for both bone marrow and peripheral blood in a congruent diagnostic category. Furthermore, all values for either D-FISH detection method that were negative for BCR-ABL fusion were also negative by CC.

**Comparison of cytogenetic analysis methodologies**

A graph of the paired studies between CC and bone marrow D-FISH with a linear regression model is shown in Figure 1. There is a single outlier, for which one subject presented at diagnosis without a t(9;22)(q34;q11.2) by CC, yet demonstrated a concurrent 92.2% BCR/ABL fusion D-FISH result in the bone marrow. Including this outlier, the correlation between the proportion of Ph-positive cells identified by CC was compared to bone marrow D-FISH with a total of 176 paired samples taken from 54 subjects. A total of three (1.70%) paired samples reflected non-congruent categorization of partial cytogenetic remission within a single paired sample. The correlation follows the relationship $y = 0.8992x + 1.6968$ where $y$ represents the proportion of BCR/ABL positive cells apparent by bone marrow D-FISH and $x$ represents CC detection of t(9;22). There is a strong correlation demonstrated by a correlation coefficient of $r^2 = 0.9203$ and $P < 0.0001$.

A graph of the paired studies between CC and peripheral blood D-FISH with a linear regression model is shown in Figure 2. The correlation between the proportion of Ph-positive cells identified through CC and peripheral blood D-FISH was explored with a total of 70 paired samples, taken from 35 subjects, of which three (4.29%) were not congruent for determination of partial cytogenetic remission status. The correlation follows the relationship $y = 0.9218x - 1.1119$ where $y$ represents peripheral blood D-FISH detection of BCR/ABL fusion and $x$ represents CC detection of t(9;22). As with CC and bone marrow D-FISH, this correlation is strong and demonstrates a correlation coefficient of $r^2 = 0.9655$ and $P < 0.0001$.

Finally, the results for the paired studies between bone marrow D-FISH detection of the Ph and peripheral blood D-FISH are shown in Figure 3. This included 50 paired samples taken from 25 subjects of which two (8.00%) were not congruent.
for determination of partial cytogenetic remission status. The correlation follows the relationship $y = 0.9675x - 1.7990$ where $y$ represents peripheral blood D-FISH and $x$ represents bone marrow D-FISH detection of BCR/ABL fusion. As in Figures 1 and 2, there is a third strong correlation between the variables demonstrated by a correlation coefficient of $r^2 = 0.9723$ and $P < 0.0001$. A summary of the correlation analysis, including measurements of correlation strength, is summarized in Table II.

**Discussion**

Several authors have previously explored the correlation between CC, bone marrow FISH and peripheral blood FISH. Mühlmann et al. [16] reported a correlation between peripheral blood and bone marrow I-FISH ($n = 30$, $r = 0.98$, $P < 0.0001$). Cuneo et al. [13] utilized a larger sample size and demonstrated a strong correlation between CC and bone marrow I-FISH ($n = 98$, $r = 0.994$, $P < 0.0001$).

![Figure 1. Correlation in CML patients between CC and bone marrow D-FISH with a linear regression model.](image1)

![Figure 2. Correlation in CML patients between CC and peripheral blood D-FISH with a linear regression model.](image2)
P < 0.0001). Lesser et al. [10] explored multiple parameters, including CC and bone marrow D-FISH (n = 35, r = 0.81, P < 0.001), CC and peripheral blood D-FISH (n = 41, r = 0.78, P < 0.001), as well as bone marrow and peripheral blood D-FISH (n = 36, r = 0.88, P < 0.04), and found less of a correlation than previously noted. Yanagi et al. [17] explored the same multiple correlations, including CC and bone marrow FISH (n = 16, r = 0.933), CC and peripheral blood FISH (n = 38, r = 0.841), and bone marrow and peripheral blood D-FISH (n = 16, r = 0.933). Yanagi et al. [17] demonstrated a stronger correlation than Lesser et al. [10], the sample sizes were reduced and there was a significant difference in the Ph proportions for each methodology.

To this end, the findings of the present study demonstrate a similar strong correlation between CC, bone marrow D-FISH, and peripheral blood D-FISH with a larger cohort and paired sample number than the previous studies reviewed above. The strength of these correlations is reflected in a P-value of < 0.0001 in all cases and a correlation coefficient of $r^2 = 0.9203$ at worst. These strong correlations are congruent with previous work, as well as the raw triplicate data listed in Table I, particularly the correlation between CC and peripheral blood D-FISH ($r^2 = 0.9655$ and $P < 0.0001$). In addition, the vast majority of paired sample sets were congruent in determining the partial cytogenetic remission status for a given subject. This indicates that, among this set of data, peripheral blood D-FISH yields a clinically equivalent Ph-detection via $BCR/ABL$ fusion to CC, particularly during the long-term management of patients with CML because most of our data is taken from follow-up visits. Furthermore, any D-FISH study, either bone marrow or blood, that indicated no evidence of $BCR/ABL$ fusion was also negative by CC. This indicates that D-FISH could also be used as a surrogate for CC in the detection of MRD and is indeed valuable for the long-term tumor burden monitoring of CML.

Each of the 296 paired samples were combined into a single data set for the purposes of statistical analysis. Because multiple paired samples were often taken from a single subject and each subject might
offer paired samples for varying correlations, these data points are not truly independent observations. Furthermore, this does not allow for correlations within a given subject and does not address the effects of differing within and between patient correlations. There is also a theoretical limitation to fitting a linear regression model to a variable constrained to lie on or between 0 and 100. Given these limitations and the strength of the associations shown, a strictly valid, more complex analysis would not yield substantially different conclusions. Furthermore, the conclusions drawn based on these data, such as determining the partial cytogenetic remission status of a patient, are dichotomous in nature and overcome these limitations.

In addition to the strong correlations noted above, the value of D-FISH in the initial diagnosis of CML, as an adjunct to CC, was highlighted by the outlier present in Figure 1. At presentation, the subject demonstrated a leukocytosis with a markedly hypercellular bone marrow for age (95%), with increased, left-shifted, granulocytic precursors, as well as increased, atypical megakaryocytes. Although diagnosed with a presumptive CML at an outside institution, the bone marrow CC yielded a normal karyotype without evidence of a t(9;22)(q34;q11.2). A concurrent FISH study was performed utilizing the bone marrow biopsy that showed 92.2% of the 500 nuclei had BCR/ABL fusion. This signal pattern observed by FISH indicated that this patient carried a cryptic insertional translocation, such that a portion of the ABL gene region was inserted into the BCR gene region of one chromosome 22. This anomaly is cytogenetically undetectable because chromosomes 9 and 22 appear to be normal due to the small molecular rearrangement occurring at the gene level. Although rare, this mechanism of BCR/ABL fusion accounts for approximately 1% of CML patients and thus is not surprising to encounter in this study of 65 patients. This emphasizes the utility of FISH in evaluating an unexplained leukocytosis, as well as its effectiveness in confirming diagnoses from outside referrals.

There is no formal standard of practice for the management of patients with CML; most agree that the use of bone marrow cytogenetics is not optimal for long-term monitoring, particularly once cytogenetic remission has been established. Tefferi et al. [18] published consensus guidelines for the application of various Ph detection methods to this effect, and there are additional guidelines currently being authored. Reverse transcription polymerase chain reaction is considered to be a surrogate for CC in Ph monitoring because it is currently the most sensitive method for Ph detection (0.001%) [18]. We propose that D-FISH from peripheral blood also has efficacy in monitoring cytogenetic remission status.

Although we advocate the substitution of peripheral blood D-FISH for disease monitoring in CML patients, it should be noted that clonal evolution occurring in any chromosomal region outside of BCR and ABL will not be detected by this FISH assay. The common additional chromosomal markers often seen in the accelerated stage of CML, including trisomy 8 and isochromosome 17q, require detection by conventional cytogenetic studies. Furthermore, if a clinical concern exists for the evolution of a therapy-related myeloid process, conventional cytogenetic studies are necessary to uncover the typical anomalies associated with myelodysplasia, such as 5q-, monosomy 7 or 20q-.

**References**


