Original article

Fluorescence In Situ Hybridization on peripheral blood is a sensitive and reliable method for evaluation of minimal residual disease in CML

Tehnica FISH efectuată pe sânge periferic este o metodă sensibilă şi de încredere în evaluarea bolii minime reziduale în leucemia mieloidă cronică

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Abstract

This study was aimed to investigate the value of detecting BCR/ABL fusion gene by fluorescence in situ hybridization (FISH) on peripheral blood specimens from patients with chronic myeloid leukemia (CML). Materials and Methods: We analyzed 126 samples (83 bone marrow and 43 peripheral blood samples) from patients with CML referred to Fundeni Clinical Institute between 2010 and 2011. Samples were analyzed using interphase FISH on peripheral-blood specimens and the results were compared with those of conventional cytogenetics and interphase FISH on bone marrow. Results: in comparison to conventional cytogenetics and bone marrow FISH, interphasic FISH on peripheral blood did not generated any discrepancies regarding the level of minimal residual disease. Conclusion: In this study we evaluated FISH technique as an alternative and rapid method for monitoring minimal residual disease in CML patients treated with tyrosine kinase inhibitors. FISH on peripheral blood is a molecular cytogenetic technique that allows minimal residual disease monitoring on interphase nuclei. This technique has the advantage of generating results in a short time (even in 24h) and is less invasive than bone marrow biopsy.

Keywords: *peripheral blood-FISH technique, BCR/ABL gene fusion, minimal residual disease*

Rezumat

Acest studiu are scopul de a evalua boala minimă reziduală prin detectarea genei de fuziune BCR/ABL prin tehnica FISH la pacienții cu leucemie mieloidă cronică. Materiale şi Metode: au fost analizate 126 de probe (83 de probe de măduvă osoasă şi 43 de probe de sânge periferic) provenite de la pacienții internați în Institutul Clinic Fundeni în perioada 2010-2011. Probele au fost analizate utilizând citogenetica convențională şi tehnica FISH. Rezultatele obținute prin citogenetica convențională şi tehnica FISH pe maduvă osoasă au fost comparate cu cele obținute prin tehnica FISH pe sânge periferic. Rezultate: în comparație cu citogenetica convențională şi

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tehnica FISH pe măduvă osoasă, tehnica FISH efectuată pe sânge periferic nu a generat discrepanțe în ceea ce privește nivelul bolii minime reziduale. Concluzie: În acest studiu noi am analizat tehnica FISH ca o metodă alternativă și rapidă a monitorizării bolii minime reziduale la pacienții cu leucemie mieloidă cronică tratați cu inhibitori de tirozin-kinază. Tehnica FISH efectuată pe sânge periferic permite evaluarea bolii minime reziduale la pacienții cu leucemie mieloidă cronică. Această tehnică are avantajul că furnizează rezultate rapid (în 24 de ore) precum și faptul că este mai puțin invazivă față de biopsia medulară.

Cuvinte-cheie: *tehnica FISH – sânge periferic, fuziune genică BCR/ABL, boală minimă reziduală*

Introduction

Chronic myeloid leukemia (CML) is the first malignancy for which a specific chromosomal abnormality was described – Philadelphia chromosome (1). Detection and monitoring of minimal residual disease (MRD) in CML is an important part of response evaluation of patients treated with tyrosine kinase inhibitors allowing early detection of suboptimal response or resistance to treatment (2).

Cytogenetic analysis is considered the gold standard for monitoring response in CML (3). Conventional cytogenetics displays many advantages over other techniques used in MRD monitoring, such as FISH or PCR, along with several limitations (3-5). The major advantage of this technique is that it allows detection of karyotypic abnormalities besides the Ph chromosome. These cytogenetic abnormalities are very useful in patient evaluation as they are frequently associated with poor prognosis or disease progression. However, additional cytogenetic abnormalities may not be detected unless they are present in 5% to 10% of cells. Also conventional cytogenetics permits additional clone identification (3).

On the other hand, 95% of patients at diagnosis have a detectable Ph chromosome, the other 5% presenting a submicroscopic insertion which is undetectable by cytogenetic analysis and should be confirmed by FISH or PCR techniques. The sensitivity of the technique is limited by the availability of metaphases and due to the fact that only 20 to 50 metaphases are being examined per patient (6, 7). These limitations of sensitivity and availability are not hindering FISH or PCR analysis. Another limitation of cytogenetics is that it is a time and labor consuming technique. Cytogenetics requires bone marrow sampling, which prevents repetitive analyses due to patient reluctance to the procedure.

FISH technique is very important in CML patient workout as it does not display many of conventional cytogenetics shortcomings. FISH assay has the advantage of higher sensitivity by allowing examination of more than ten times the number of cells analyzed by conventional cytogenetics (8). Due to its higher sensitivity it permits detection of BCR-ABL fusion gene in Ph chromosome negative patients (undetectable in cytogenetics analysis). FISH enables a rapid detection of chromosomal rearrangements on metaphases or interphase nuclei. In this latter case, cell cultures are no longer necessary. Moreover, this technique allows the examination of peripheral blood cells (PB) when bone marrow (BM) sampling is not available. However, FISH on PB samples has a potential limitation in sensitivity as the majority of the cells in periphery are Ph negative compared to BM (9, 10). Also, in a minor proportion of the patients treated with tyrosine kinase inhibitors FISH analysis of BM may yield lower Ph positive percent than conventional cytogenetic analysis (11, 12).

New generation of FISH probes use a dual fusion and even triple fusion format which improves specificity and sensitivity of the assay (11). FISH analysis can be used to monitor CCyR (Complete Cytogenetic Response), although standard LeukemiaNet recommendations are reserved in this regard (13, 14).

In this study we examined FISH technique as a rapid and non-invasive alternative method for MRD monitoring in CML patients undergoing tyrosine kinase inhibitor treatment. We also analyzed

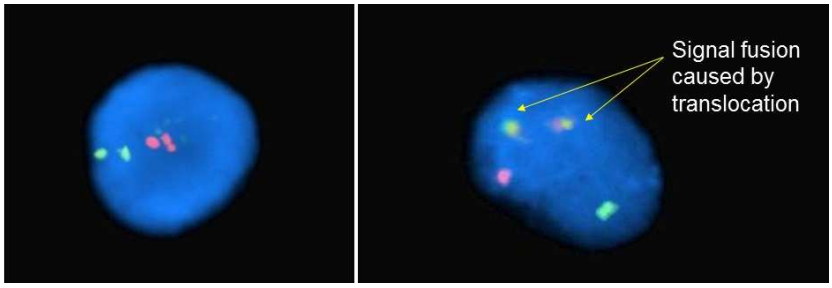

Figure 1. A normal cell with two clearly separated Green (BCR probe) and Red (ABL probe) signals (left); a Ph-positive cell with one Red, one Green, and two Yellow signals (fusion of the ABL and BCR probes on the Ph chromosome) (right)

the concordance of the results of conventional cytogenetics, BM FISH and PB FISH.

Material and Methods

Patients and samples

A total of 83 samples of BM and 43 samples of PB from 83 patients with CML referred to Fundeni Clinical Institute were included in the study. 31 samples of BM and 21 of PB derived from patients at the onset of the disease and 52 samples of BM and 22 samples of PB derived from patients during the course of therapy. In all cases, heparin-anticoagulated fresh bone marrow (BM) aspirate samples and PB were obtained and used for the cytogenetic examination.

Conventional Karyotyping

Bone marrow samples were cultured using overnight and synchronized culture and processed by conventional cytogenetic procedures with GTG banding (15). In each case, at least 20 metaphases were analyzed and the karyotypes were described according to ISCN 2009 (16).

FISH Analysis

FISH analysis was performed on interphase cells using a dual-color BCR/ABL probe, provided by Cytocell, Cambridge, UK (*Figure 1*).

Slides for FISH analysis from peripheral blood were obtained as follows: 1 mL of peripheral blood was mixed with 9 mL of RBC buffer (Red Blood Lysis Buffer) (Qiagen). Cells were kept on ice for 10 minutes and centrifuged for 10 minutes at 1500 rpm. Supernatant was aspirated and cells washed once in 1x PBS buffer and centrifuged for 10 minutes at 1500 rpm. Supernatant was aspirated and cells were incubated in hypotonic KCl solution (0.075M) for 10 minutes at 37^0C and then fixed in methanol: acetic acid - 3:1 for three times. After slide preparation, cells were aged by incubation overnight at 37^0C. FISH analysis of cultured BM samples was performed using slides obtained by usual protocol for cytogenetic examination. Slides were dehydrated an alcohol series. Co-denaturation was carried out for 2minutes at 75^0C, followed by overnight hybridization at 37^0C. After overnight hybridization slides were washed in 0,4X SSC at 73^0C for 2 minutes and rinsed in 2 X SSC. Evaluation of the FISH signals was performed using a fluorescence microscope (AxioImager, Zeiss, Germany). For each case, a minimum of 200 interphase nuclei were evaluated.

Results

The analysis was performed on the 126 samples (83 BM samples and 43 PB samples) from patients with CML treated with tyrosine kinase inhibitors. 45 BM samples exhibited Ph chromosome on standard karyotype and, as expected, two dual fusion signals in FISH analysis on BM. 38 samples were negative in both analyzes. Of PB samples 12 were negative for Ph+ and 31 positive.

Comparison between Cytogenetics and Interphase FISH on BM Specimens

The analysis was performed on 83 bone marrow specimens. A very good correlation was found between conventional cytogenetics and interphase FISH on BM (*Figure 2A*). The only discrepancies between cytogenetic analysis and FISH on BM observed were related to Ph chromosome percentage. In this case, FISH analysis, generally, exhibited a higher Ph+ percentage.

Comparison between Cytogenetics on BM and Interphase FISH on PB Specimens

PB samples were available only in 43 cases and comparative analysis with cytogenetics also exhibited a high level of correlation of the results (*Figure 2B*). For 2 samples we found a significant difference between PB FISH results and cytogenetics relating to Ph+ percentage. Moreover, 3 specimens from patients in major response by cytogenetics were classified in the complete response group by FISH on PB.

Comparison between Interphase FISH on BM Cells and on PB Cells

Comparison of FISH analyzes performed on 43 specimens of bone marrow and blood specimens collected simultaneously, revealed a good correlation, with a slightly lower percentage of Ph+ cells indentified by PB FISH. As with cytogenetic analysis, the same 3 samples which tested positive on BM samples were negative on PB FISH analysis. Also the same 2 samples which presented a significant difference in Ph+ percentage on cytogenetic analysis exhibited a significant difference in this comparison. These discrepancies are attributable to different dynamics of leukemia cells in BM and PB. Results are displayed in *Figure 3.*

In 8 cases we observed abnormal FISH patterns confirmed by standard cytogenetics: loss of terminal region of derivative chromosome 9, loss of derivative chromosome 9 and supernumerary Ph.

Discussion

Recent studies have shown the feasibility and accuracy of FISH analysis for MRD monitoring in CML patients (17-19). Results of these studies suggest that the dual fusion probe format is very convenient and allows identification of all possible fusion types. Moreover these probes allow identification of cryptic insertion of BCR-ABL fusion gene undetectable by standard cytogenetics (8, 17, 20). FISH analysis displays higher sensitivity than standard cytogenetics due to the possibility of examination of as many as 200 nuclei (or more) per sample, which enables MRD detection at lower then 1% level (17). The use of interphase nuclei makes this technique especially valuable in case no or few metaphases are obtained (13).

Detection of BCR-ABL in PB by I-FISH has therefore been proposed as an alternative to conventional cytogenetics. In most direct comparisons, the two methods showed a good correlation in 3 individual studies (17, 19 and 20).

In our study we proposed the implementation of a method of MRD monitoring using PB-FISH. This method allows the examination of PB leukocytes for BCR-ABL fusion gene and does not require BM aspiration. BM aspiration is an invasive technique and many patients are reluctant to perform it especially on regular basis during therapy, which hinders proper MRD evaluation. Other advantages of the technique include lower cost and lack of culture artifacts due to the fact that cell are prepared using the direct method. Also, this technique has lower turn-around time and results are potentially provided in one business day.

To demonstrate the feasibility and accuracy of PB-FISH, in this study we performed the following analyzes: 1 comparison of conventional cytogenetics and interphase BM-FISH; and 2 comparison of interphase PB-FISH and BM-FISH for measuring MRD level.

Comparison of BM-FISH and standard cytogenetics was performed on 83 samples and results show that, in most of the cases, interphasic BM-FISH exhibited small differences of BCR/ABL positive nuclei levels. Comparison of PB-FISH with conventional cytogenetics was performed on 43 samples. As seen in *Fig-*

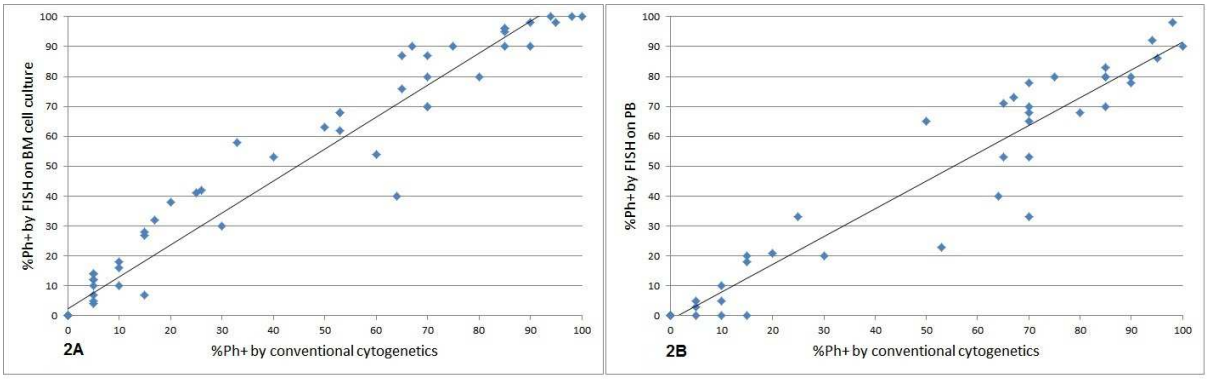

Figure 2A. Correlation between conventional cytogenetics results (x-axis) and interphase FISH (y-axis) on bone marrow specimens. 2B. Correlation between conventional cytogenetics (x-axis) and interphase FISH (y-axis) on periferal blood specimens

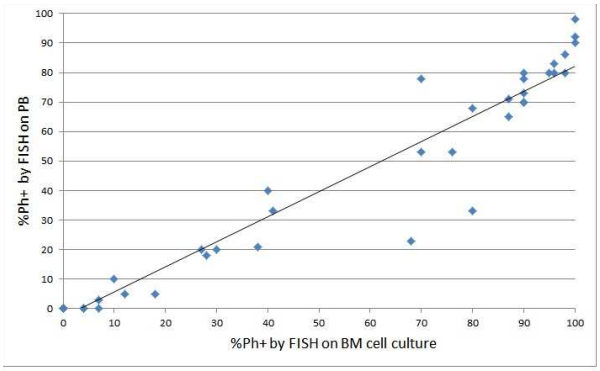

Figure 3. Correlation between interphase FISH results on bone marrow (x-axis) and peripheral-blood (y-axis) specimens

ure 1, a high degree of correlation was obtained although a generally smaller percentage of Ph chromosomes was identified by PB-FISH, which partially reflects higher abundance of leukemia cells in BM. Discrepant results observed here are also related to lower leukemia cell percentage in PB – in 3 samples no Ph+ nuclei and in 2 significantly lower percentage of Ph+ nuclei were identified in PB FISH analysis as compared to standard cytogenetics.

BM and PB-FISH comparison revealed a slightly higher percentage of Ph chromosomes in BM-FISH versus PB-FISH, an observation consistent with standard cytogenetics results (*Figure 2*). Discrepant results between standard cytogenetics and PB FISH were also found in this comparison. These discrepancies are attributable to different leukemia cell loads in BM and PB and were also described in a number of previous studies (17, 19 and 20).

Overall, PB-FISH exhibited high degree of correlation with BM-FISH (with the exception of 5 samples which presented significant discrepancies in Ph+ percentage) and standard cytogenetics results which makes this technique very useful for MRD monitoring of CML patients, especially of patients in CCyR or long term CcyR, as it is rapid, accurate and relatively non-invasive.

Conclusions

PB-FISH is a molecular cytogenetics technique that allows MRD monitoring on interphase nuclei in CML patients. MRD monitoring on peripheral blood has the advantage of generating results in a short time (even in less than 24h) and is less invasive than bone marrow biopsy. Overall, this method exhibited very good correlation with BM-FISH and standard cytogenetics results and is an accurate and sensitive alternative method for monitoring MRD in CML patients in CCyR and long term CCyR.

Abbreviations

BM- Bone Marrow
CCyR- Complete Cytogenetic Response
CML- Chronic Myeloid Lekemia
FISH- Fluorescence In Situ Hybridization
ISCN- International System for Human Cytogenetic Nomenclature
MRD- Minimal Residual Disease
PB- Peripheral Blood
PCR- Polymerase Chain Reaction
Ph chromosome – Philadelphia chromosome
RBC - Red Blood Lysis Buffer

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