

Case Report

Fluorescence *in situ* hybridization on Peripheral blood for Chronic Myeloid Leukaemia - Rapid and reliable method

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Abstract

Philadelphia chromosome (Ph) is found in more than 95% of Chronic Myeloid Leukaemia (CML) patients arising from the reciprocal translocation of chromosomes 9 and 22 which results in the formation of chimeric fusion gene BCR-ABL. This paved the path for targeted gene therapy in CML and thus plays a pivotal role in diagnosis and prognosis. Fluorescence *in situ* hybridization (FISH) is a rapid and reliable technique in molecular cytogenetics to detect BCR-ABL fusion signal in both interphase and metaphase spreads of bone marrow sample. Peripheral blood white cells as a surrogate for bone marrow have been suggested by a few studies.

The objective of the study was to evaluate FISH on peripheral blood specimen as a rapid and reliable method to quantify Ph positive cells in a patient with Chronic Myeloid Leukaemia. FISH was performed on interphase nuclei from cultured peripheral blood sample of the patient using BCR/ABL Translocation, Dual fusion probe. Chromosomal analysis was performed by GTG banding technique. FISH and karyotyping confirmed the presence of reciprocal translocation t(9; 12)(q34.1; q11.2).

Our results confirmed that FISH technique is a rapid, sensitive, quantitative technique which can be used for the evaluation of CML using peripheral blood. FISH helps in the detection of minimal residual disease and disease recurrence with small percentage of abnormal cells. In our experience, this situation is usually associated with very high WBC count which can result in increase in the percentage of Ph-positive cells.

Keywords: Fluorescence *in situ* hybridization (FISH), chromosomal analysis, chronic myeloid leukemia, peripheral blood.

Introduction

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder resulting from neoplastic transformation of haemopoietic stem cells that can lead to increase of myeloid series in peripheral blood and myeloid hyperplasia in bone marrow. The most common adult leukemia in India is CML with annual incidence ranging from 0.8–2.2/100,000 population in males and 0.6–1.6/100,000 population in females [1].

The discovery of the first chromosomal abnormality in CML was done by Nowell and Hungerford in 1960 in Philadelphia and the deletion of one of the

shortened chromosome of chromosomes 22 pair was named as 'Philadelphia chromosome' to honor the city [2]. The presence of Philadelphia chromosome (Ph) differentiates the chronic myeloid leukaemia from other chronic myeloproliferative disorders [3].

Philadelphia chromosome arising from the reciprocal translocation between long arm of chromosomes 9 and 22 that fuses the ABL (Abelson Murine leukemia Virus) oncogene on chromosome 9 to the BCR (breakpoint cluster region) gene on chromosome 22. This results in the formation of chimeric fusion gene BCR-ABL and this translocation - t(9;22)(q34;q11.2) was identified with advancement of banding techniques which became the diagnostic hallmark of CML. Conventional cytogenetics or karyotyping is the standard method to detect

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chromosomal aberration. In addition to Ph chromosome, chromosomal abnormalities like trisomy 8 and isochromosome 17 also can be identified in CML cases by using chromosomal analysis [3].

Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetics technique, becoming a vital part of clinical practice in the workup of patients with hematological malignancies. FISH can be used to investigate both proliferating (metaphase cells) and non-proliferating (interphase nuclei) cells by detecting the location of specific nucleic acid sequences, using fluorescently labeled specific DNA probes to the target sample. FISH has been used as the most reliable method to identify the BCR/ABL gene at the time of diagnosis [3].

Materials and Methods

Subject

A 52 year old female presented with fatigue and fever of one month duration was admitted in K.S. Hegde Charitable hospital, Deralakatte, Mangalore. On examination, she had pallor and splenomegaly 4cms below the costal margin. The blood investigations revealed total WBC count of $2.5 \times 10^9/L$ and hemoglobin level of 9gms/dl. Peripheral blood smear confirmed the diagnosis of CML. (Differential count - 14 nRBCs/100 WBC, Myeloblast - 11%, Promyelocytes - 7%, Myelocytes - 21%, Metamyelocytes - 13%, Band forms - 12%, Neutrophils - 23%, Eosinophils - 8%, Basophils - 5%.) Peripheral blood smear picture of the patient is shown in Figure 1 & 2.

Owing to her high WBC count, 3ml of peripheral blood was collected in sodium heparin vacutainer tube and sent for karyotyping and Fluorescence *in situ* hybridization (FISH) to Diagnostic Centre for Cytogenetics and Molecular genetics.

Conventional cytogenetic analysis

Direct and 24 hour cultures were set up with peripheral blood using RPMI 1640 medium supplemented with 20% of fetal bovine serum. GTG banding was done using standard cytogenetic protocol. Metaphases were analyzed from each culture, captured using the Olympus BX53

microscope and karyotyped with the 'GENASIS' software. Ph chromosome positivity was expressed in all 20 metaphases analyzed and the karyotype was reported according to An International System for Human Cytogenetic Nomenclature (ISCN) 2013.

Fluorescence in situ hybridization (FISH) Analysis

FISH analysis was carried out on interphase cells and metaphases of the fixed cell pellet using BCR/ABL Translocation, Dual fusion probe (Cytocell, UK). The BCR/ABL probe set comprises a BCR probe mix directly labeled in green (Spectrum Green) spanning at 22q11.22-q11-23 and the ABL1 probe directly labeled in red (Spectrum Orange) spanning at 9q34.11-q34.12. Cell pellet was dropped on the slide and 10 μ l of probe was applied to the target area. The sample and probe were co-denatured and hybridized using the ThermoBrite Denaturation /Hybridization System.

The ThermoBrite unit was programmed to allow 5 minutes of denaturation at 73°C to transform the double strands DNA into a single strand, followed by overnight hybridization at 37°C for the probe to hybridize with the target cells and bind to it. Next day, the slide was washed with 0.4X SSC/0.1% NP-40 at 72°C for 2 minutes, followed by a wash in 2X SSC/0.1% NP-40 at room temperature for 1 minute to remove any of the probes that did not bind to the cells. The slide was air dried in the dark, then added 10 μ l of DAPI (4, 6-diamidino-2-phenylindole) as a counter stain.

The FISH signals were visualized using an Olympus BX53 fluorescence microscope equipped with appropriate filters. Interphase nuclei and available metaphases were scored and the signals were captured using a CCD camera attached to a FISH View image acquisition and analysis system for FISH (GENASIS, Applied Spectral Imaging, Germany).

Results

Cytogenetic analysis of a metaphase showing 46,XX,t(9;22)(q34;q11.2) karyotype, which results in the Philadelphia chromosome is shown in Figure 3. FISH analysis of normal interphase nuclei will have a simple 2R, 2G signal pattern.

As a result of the Philadelphia chromosome, ABL1 (Abelson) proto-oncogene and the BCR (Break point Cluster Region) gene fuse, giving rise to the BCR/ABL1 fusion gene showed 1 green, 1 red and 2 yellow fusion signals (1G, 1R, 2Y) in all the cells analysed (Figure 4 & 5).

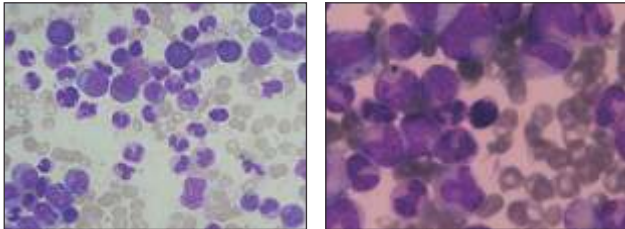


Figure 1 & 2 : Peripheral blood smear – Leishman's stain x400 and x 1000 Observe increased WBC count and the presence of increased myeloid series cells.

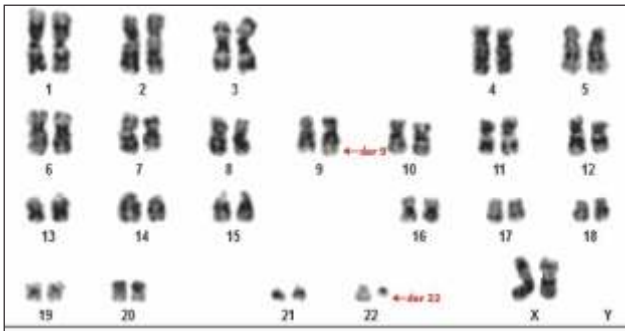


Figure 3 : GTG-banded karyogram of the patient showing 46,XX,t(9;22)(q34;q11.2)

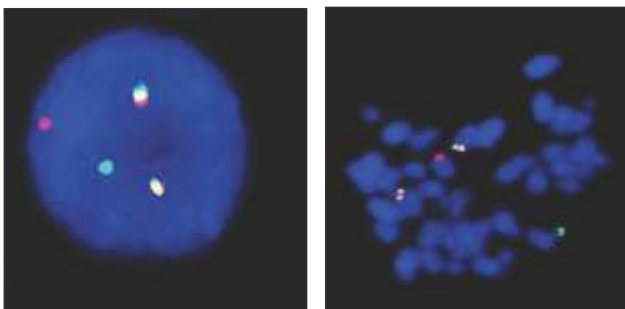


Figure 4 & 5 : Representative interphase cell and metaphase showing BCR/ABL fusion as yellow signals, which represents overlapping of BCR (green) and ABL (red) signals. The other red signal represents ABL on chromosome 9 and green represents BCR on chromosome 22.

Discussion

Ph chromosome is present in 95% of CML cases and also present in 5 % of children and 20% of adults with acute lymphoblastic leukemia (ALL) [3]. Conventional cytogenetics is considered as the diagnostic hallmark for

identification of Ph positive metaphases in CML cases when it is present in more than 30% cells. This method has its own limitations like increased turn around time, labour intensive procedure and analysis dependent on high numbers of viable dividing cells and well spread metaphases. Reena *et al*/studied the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of conventional karyotyping, reported as 85%, 100%, 100% and 71% respectively. The accuracy for conventional karyotyping method was 88% [3]. In the present study, conventional cytogenetics was done with 20 metaphase spreads and the Philadelphia chromosome (Ph+) was seen in all well spread metaphases. Good metaphase spreads are difficult to get and may consume extra time for analysis. However, conventional cytogenetics has its strength of detecting additional nonrandom chromosomal aberrations within the 20 spreads [4].

The quantification of BCR-ABL positive cells by FISH facilitates the monitoring of disease response to therapy [2]. The accuracy of FISH in the analysis of *BCR-ABL* rearrangements of CML patients has been demonstrated by several recent studies [5,6,7]. Even 1-2% of positive cells can be picked up due to the sensitivity of FISH test [5]. FISH was carried out in this patient with 200 interphase nuclei and available metaphases. The presence of Ph chromosome was confirmed and proved the accuracy of FISH on peripheral blood which was 100%. This technique has many advantages, which include analysis of large number of cells, speed of reporting within 24 hours of the sample received in the laboratory, which may be useful in urgent diagnostic situations. It can be performed with fixed cell pellets left over from cytogenetic study of bone marrow or peripheral blood and paraffin embedded tissues [3].

Le Guill *et al* studied the correlation between FISH and cytogenetics on bone marrow and got highly correlated results, then they moved on to the analysis of peripheral blood samples where comparable results were obtained, with an excellent correlation ($r = .97$) [6]. Earlier reports

have demonstrated that FISH technique was able to detect all types of *BCR-ABL* fusions in CML with masked or variant Ph, which is not apparent with the conventional karyotyping [6,8]. Lim *et al* observed additional karyotypic changes like supernumerary Ph or deletions of 9q and/or 22q in 28.1% of Ph positive patients by interphase FISH [8]. The detection of *BCR-ABL* cells by Interphase FISH correlates better with molecular response, the exact interpretation of FISH in conjunction with chromosomal analysis is the best practice, to avoid the possibility of misdiagnosis [8,9].

Patel *et al* observed significantly reduced disease free survival in patients with deletion in ABL, BCR or ABL, BCR on derivative 9 as compared to non-deleted derivative 9 and their findings suggest that the gene segments around the breakpoint are deleted in heterogeneous patterns when the translocation occurs and the genes located near the breakpoint may also play a role in the delayed tumor progression [10]. Thus, FISH technique gives information about the phases of CML and useful at diagnosis after BMT

(Bone Marrow transplantation) for detection of residual diseases.

Conclusion

Chromosomal analysis and FISH both have their advantages and should ideally be carried out together in order to make a correct diagnosis and predict/monitor response to newer molecular targeted treatment modalities like imatinib mesylate. FISH can be done in peripheral blood when the WBC count is high which can result in increase in percentage of Ph positive cells and also failed cases of conventional cytogenetic analysis to quantify disease in CML. FISH on peripheral blood exhibited a high degree of correlation with bone marrow, as a rapid, accurate and relatively non-invasive method.

Acknowledgement

The authors wish to thank the patient for providing the sample and written informed consent form (ICF). The authors are also grateful to Nitte University Ethics Committee for publication approval and to Mrs. Vani Shetty for her technical help.

References

1. Subramanian PG. Cytogenetic study in CML. Indian J Med Res. 2012;135:12-13.
2. Jha CB, Kucheria K, Choudhary VP. Diagnostic role of conventional cytogenetics and fluorescence in situ hybridization (fish) in chronic myeloid leukemia patients. Kathmandu University Medical Journal. 2006;4(2):171-175.
3. Reena RMZ, Julia Munchar MJ, Salwati S, Zubaidah Z, Hamidah NH, Sharifah NA, Cheong SK. Detection of BCR/ABL Gene in Chronic Myeloid Leukaemia: Comparison of Fluorescence *in situ* Hybridisation (FISH), Conventional Cytogenetics and Polymerase Chain Reaction (PCR) Techniques. Med & Health. 2006;1(1):5-13.
4. Anand MS, Varma N, Varma S, Rana KS, Malhotra P. Cytogenetic & molecular analyses in adult chronic myelogenous leukaemia patients in north India. IJMR. 2012;135:42-48
5. Madon P, Athalye A, Bandkar V, Dhupal S, Sopariwala A, Parikh F. Fluorescence *in-situ* Hybridization (FISH) – A Rapid and Useful Technique for Diagnosis and Management in Leukemia. Int J Hum Genet. 2003;3(2):115-119.
6. Le Gouill S, Talmant P, Milpied N, Daviet A, Ancelot M, Moreau P, Harousseau JL, Bataille R, Avet-Loiseau H. Fluorescence In Situ Hybridization on Peripheral-Blood Specimens Is a Reliable Method to Evaluate Cytogenetic Response in Chronic Myeloid Leukemia. Journal of Clinical Oncology. 2000;18(7):1533-1538.
7. Landstrom AP, Ketterling RP, Knudson RA, Tefferi A. 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. Leukemia & Lymphoma. 2006;47(10):2055-2061. DOI: 10.1080/10428190600783551
8. Lim TH, Tien SL, Lim P, Lim AST. The Incidence and Patterns of BCR/ABL Rearrangements in Chronic Myeloid Leukaemia (CML) Using Fluorescence *in-situ* Hybridization (FISH). Ann Acad Med Singapore. 2005;34:533-538.
9. Testoni N, Marzocchi G, Luatti S, Amabile M, Baldazzi C, Stacchini M, *et al.*. Chronic myeloid leukemia: a prospective comparison of interphase fluorescence in situ hybridization and chromosome banding analysis for the definition of complete cytogenetic response: a study of the GIMEMACMLWP. BLOOD. 2009;14(24):4939-4943. doi:10.1182/blood-2009-07-229864.
10. Patel BP, Trivedi PJ, Brahmabhatt MM, Gajjar SB, Iyer RR, Dalal EN, Shukla SN, Shah PM. Deletion of derivative ABL, BCR or ABL-BCR fusion gene is associated with shorter disease free survival in CML patients. Int. J. Genet. Mol. Biol. 2010;2(7):158-164.