

Fluorescence *in-situ* Hybridization (FISH) – A Rapid and Useful Technique for Diagnosis and Management in Leukemia

Prochi Madon, Arundhati Athalye, Vijay Bandkar, Suresh Dhumal,
Arifa Sopariwala and Firuza Parikh

Department of Assisted Reproduction and Genetics, Jaslok Hospital and Research Centre, 15, Dr. G. Deshmukh Marg, Mumbai 400 026, Maharashtra, India Fax: 4950508 E-mail: prochimadon@hotmail.com

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ABSTRACT Fluorescence *in situ* hybridization (FISH) is a rapid reliable technique in molecular cytogenetics. It supplements conventional karyotyping by providing additional information in certain cases. A large number of cells are available for quantitative analysis by FISH, as even interphase nuclei can be studied. It helps in detection of minimal residual disease and disease recurrence, as a very small percentage of abnormal cells can also be identified. The FISH probes used in this study were for the detection of the BCR/ABL fusion or t(9;22) in chronic myeloid leukemia (CML), PML/RARA fusion or t(15;17) in acute promyelocytic leukemia (APML) and determination of the XX/XY ratio in sex mismatched bone marrow transplantation. One hundred and fifty eight heparinized bone marrow or leukemic blood samples referred by consultants were analysed using the above mentioned FISH probes. These included samples of patients who underwent bone marrow transplantation. Karyotyping was carried out where indicated. Cases where FISH provided additional information or a different interpretation to karyotype analysis have been described.

INTRODUCTION

Fluorescence *in-situ* hybridization (FISH) is a rapid diagnostic test using molecular cytogenetic techniques. It has wide applications in many branches of medicine including oncology. The FISH technique supplements conventional cytogenetics and in some cases provides additional information, which is not detected by karyotyping. A large number of cells can be studied by FISH, since interphase nuclei can also be analyzed. This helps in the detection of minimal residual disease, assessment of the rate of cytogenetic remission and detection of disease recurrence (Amare et al. 2001).

In leukemia the commonly used FISH probes which help in diagnosis and management, identify

the BCR/ABL fusion gene in chronic myeloid leukemia (CML) and the PML/RARA fusion gene in acute promyelocytic leukemia (APML). These fusion genes are the molecular consequences of the translocation t(9;22) characterized by the Philadelphia chromosome in CML and the translocation (15;17) in APML. These fused transcripts are endowed with tumorigenic properties (Kucheria et al. 1999). The abnormal BCR/ABL tyrosine kinase gene product has enhanced activity compared to the wild type ABL tyrosine kinase, and is believed to be central to the pathogenesis of the disease (Cohen et al. 2001).

The Philadelphia chromosome (Ph) is the cytogenetic hallmark of CML and is observed by karyotyping in approximately 95% of CML patients, who are termed Ph positive (Ph+) (Champlin et al. 1985). In less than 10% of patients, additional chromosomes are involved in the translocations and these are called variant Ph translocations (Heim et al. 1985). A minority, (less than 5%), of CML patients show no Ph chromosome cytogenetically, and are called Ph negative (Ph-). In half of the Ph-CML, the BCR/ABL fusion gene is detected at the molecular level and its localization can be visualized microscopically, by FISH with dual colour BCR/ABL probes (Terre et al. 2001). In most variant Ph+ or Ph-CML's, the BCR/ABL hybrid gene has been localized either on the Ph chromosome or on one of the apparently normal chromosomes 22. In rare cases, the BCR/ABL fusion gene has been localized on 9q34 instead of 22q11 (Terre et al. 2001).

Chronic myeloid leukemia is a biphasic disease with an initial chronic phase that is readily controlled. However, this is followed by an ill-defined accelerated phase and then a terminal blastic phase that resembles an acute leukemia, which is usually refractory to therapy. Transformation to blast crisis is usually accom-

panied by secondary cytogenetic changes, which can be detected by karyotyping. Treatment options for patients with chronic phase CML currently include hydroxyurea, interferon alpha with or without cytosine arabinose and allogeneic or autologous stem cell transplantation (Huntly et al. 2001). Recently there has been considerable interest in STI 571, a novel agent specifically designed to inhibit the tyrosine kinase activity of BCR/ABL, which has shown excellent early results in all phases of the disease (Druker et al. 2001 a, b). Recent studies in APML have shown that a combined treatment modality using all-trans-retinoic-acid (ATRA) plus chemotherapy and/ or arsenic trioxide in t(15;17) positive patients confers a significant improvement in disease free survival (Advani et al. 1999). An important goal therefore is the identification at diagnosis of patients who have a poor prognosis and give them more intensive treatment to improve their outcome. Good prognosis patients are thus spared unnecessary treatment (Bain 2001).

The FISH technique can identify isolated abnormal cells among a large group of normal cells and is therefore used for quantitative analysis to monitor the haematologic response to treatment. Another advantage of FISH is that it can detect subtle translocations like t(15;17) which cannot always be detected by conventional cytogenetics. In CML, masked/ variant Ph chromosomes with submicroscopic rearrangements can be missed by karyotyping, but are easily detected by FISH (Madon et al. 2002). This demonstrates the importance of using both routine cytogenetics and FISH for a precise diagnosis and subsequent treatment of patients.

In the present study FISH was carried out with or without karyotyping on 158 samples for detection and quantitative analysis of t(9;22), t(15;17) or determination of XX/XY ratio in cases of sex mismatched bone marrow transplantation (BMT).

MATERIALS AND METHODS

Patient Samples: One hundred and fifty eight heparinised bone-marrow or leukemic blood samples of patients diagnosed or suspected of having either CML or APML were sent by consultants for FISH alone, or FISH and karyotyping. Of these, 110 samples were sent for detection of the BCR/ABL fusion and 12 samples for XX/XY ratio in CML patients and 36 samples

were sent for detection of the PML/RARA fusion in APML. In 21 of these patients, FISH/ karyotyping was repeated serially 2-6 times (60 samples) to monitor their treatment.

FISH Probes and Detection Systems: FISH was performed using directly labeled dual colour LSI/CEP probes from Vysis for detection of BCR/ABL and PML/RARA genes or XX/XY ratio in BMT. The main steps in the methodology include hypotonic treatment and fixation of direct demecolcine treated cultures, followed by dehydration, codenaturation, overnight hybridization, washing and mounting of slides in DAPI-antifade.

The slides were viewed on a Zeiss Axioskop 2 microscope with a HBO 100 mercury lamp and a spectrum green-spectrum orange dual filter. The signals were captured using a CCD camera, single filters and the Metasystems 'isis' FISH software. For quantitative analysis, 100 – 500 nuclei and available metaphases were scored. Normal cells without the fusion gene showed 2 green and 2 orange signals (Fig. 1/a.). Cells with the BCR/ABL or PML/RARA fusion gene showed 1 green, 1 orange and either 1 yellow fusion signal or overlapping green and orange signals (Fig. 1/b.). To determine the XX/XY ratio in BMT, XX cells showed 2 green signals, while XY cells showed 1 green and 1 orange signal using Vysis Aneuvision CEP probes. Chromosome 18's with 2 aqua signals are included in this probe set (Fig. 1/ c.).

Karyotyping: Multiple (direct, 24, 48 and/or 72 hour) cultures were set up in each case using RPMI 1640 medium supplemented with serum. Harvesting, fixation, slide preparation and GTG banding was done using standard protocols. Metaphases were analyzed from each culture. The images were captured using the Zeiss microscope and karyotyped with the 'ikaros' software. A metaphase showing t(9;22)(q34;q11) which results in the Philadelphia chromosome is shown in Figure 2a.

RESULTS AND DISCUSSION

Of the 110 CML samples referred, 45 were for FISH and karyotype analysis, 31 for analysis only by FISH and 34 only by karyotyping, as FISH was not available in the earlier part of the study. All the 45 samples referred for FISH and karyotyping were Ph+ by FISH with a varying percentage (3-100%) of Ph+ and normal cells,

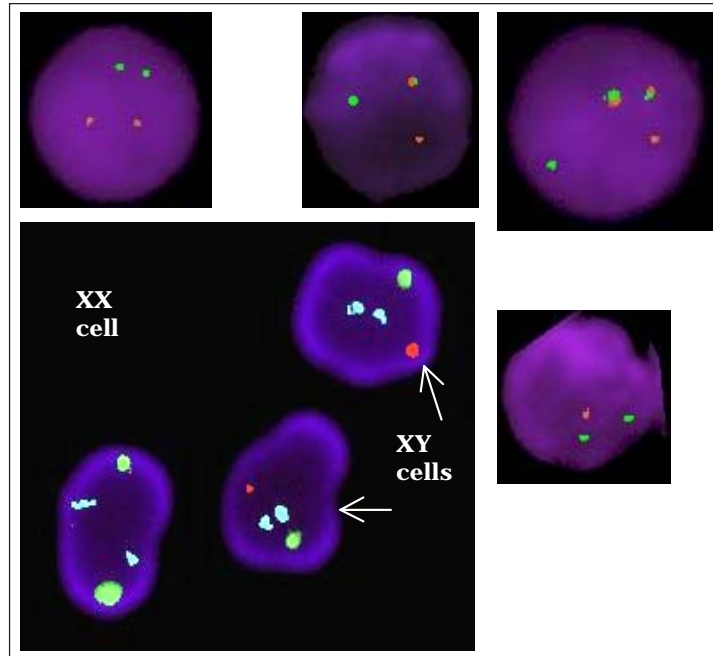


Fig. 1. Fluorescence in situ hybridization (FISH)

- Normal cell with BCR/ABL probe shows 2 green (BCR) and 2 orange (ABL) signals.
- Ph⁺ cell with BCR/ABL probe shows 1 green, 1 orange and 1 yellow or overlapping orange and green signal indicating fusion.
- FISH using CEP probes for chromosomes X, Y and 18 to determine % of XX (2 green – chr. X and 2 aqua – chr. 18) and XY (1 green – chr. X, 1 orange – chr. Y and 2 aqua – chr. 18) cells.
- FISH in a CML patient (Case 1) showing 2 BCR/ABL fusion signals.
- FISH showing a single orange signal indicating deletion of the ABL locus on 1 allele in 95% cells in acute leukemia (Case 5).

but only 17 were Ph⁺ by karyotyping. The samples which were Ph⁻ on karyotyping were those with a low percentage (3-20%) of Ph⁺ cells on FISH, except 3 cases which had variant/masked Ph chromosomes. Some interesting cases illustrating the importance of FISH together with karyotyping for an accurate diagnosis are described below.

Case 1: A patient with CML was Ph⁺ by FISH. Two BCR/ABL fusion signals were observed in 90% cells (Fig. 1d). Karyotyping showed 2 Ph⁺ chromosomes in 65% cells but there was a translocation t(12;22)(p13;q11) instead of t(9;22)(q34;q11). The derivative 22 looked like the Ph⁺ chromosome but the derivative 9 was absent as the part of chromosome 22q which is usually translocated to 9q, was located on 12p (Fig. 2b). Hence this was a case of a complex translocation t(9;22;12)(q34;q11;p13) which could only be delineated by karyotyping together with FISH.

Case 2: A case of CML was 100% Ph⁺ by FISH, but Ph⁻ on karyotyping. FISH on metaphases revealed that the BCR/ABL fusion signal was localized on 9q34 instead of 22q11. This has been reported in very rare cases (Terre et al. 2001). Isochromosome 17q was present in 1 clone together with additional markers in another clone of cells.

Case 3: Karyotyping in a case of CML showed t(7;22)(q11;p11) and a Ph⁻ status. However, FISH showed the BCR/ABL fusion in 100% cells, indicating the presence of a masked Ph.

Case 4: A patient with CML was Ph⁺ on karyotyping of leukemic blood and FISH analysis. However the derivative 9 looked different, as the translocation seemed to involve 9p instead of 9q. Two clones involving 1 or 2 derivative 17's were also present, probably indicating progression of the disease.

Case 5: A patient with acute leukemia had a normal karyotype. FISH however showed 95%

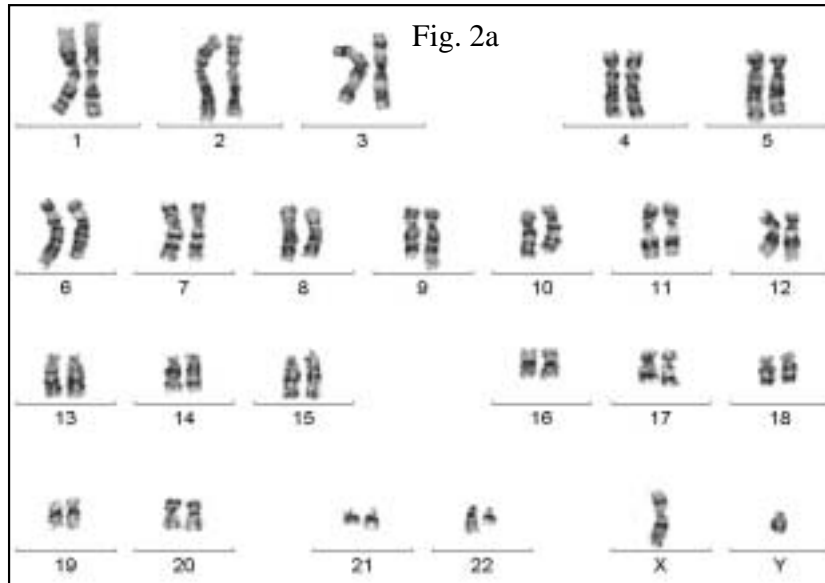


Fig.2 a. Karyotype of a CML patient showing translocation $t(9;22)(q34;q11)$.

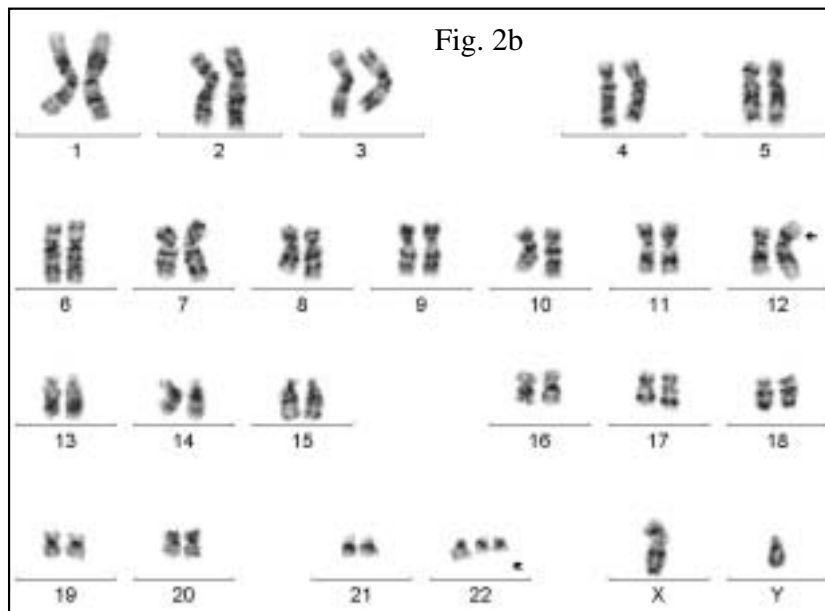


Fig.2 b. Karyotype of Case 1 showing a complex translocation $t(9;22;12)(q34;q11;p13)$ with double Ph⁺ chromosomes. The chromosome 9's looked normal on karyotyping, but FISH showed double BCR/ABL fusion signals in 90% cells.

cells with only 1 orange signal indicating deletion of the ABL locus on 1 allele (Fig. 1e).

Case 6: A case of MDS showed a Ph+ karyotype. As this was unlikely, FISH was done to confirm the finding, but the BCR/ABL fusion signal was absent. Instead, a deletion of 1 green signal was seen in all the cells, indicating a deletion of part of chromosome 22q involving the BCR locus 22q11. This explained the derivative chromosome 22 seen on karyotyping, which was mistaken for the Philadelphia chromosome.

Among the CML cases karyotyped, clonal evolution was detected in 9 patients. Double Philadelphia chromosomes were also observed. On FISH, double fusion (only 2 fusion signals without single green and orange signals) was occasionally observed. In addition, deletion/amplification of the BCR and/or ABL regions was observed in 5-15% nuclei on FISH in a few cases. This probably indicates progression of the disease.

Of the 36 samples referred for APL detection, 11 were for FISH and karyotyping, 20 were referred only for FISH analysis all of which were positive and 5 were only karyotyped when FISH was not available. The subtle translocation t(15;17) could not be detected by karyotyping in these 5 cases. Of the 11 samples where FISH and karyotyping was done, all were positive in varying percentages by FISH, but only 5 were positive by karyotyping. The translocation could be detected by karyotyping only when it was present in more than 30% cells by FISH. The FISH test was very sensitive as it could pick up even 1-2% positive cells.

Karyotyping and FISH both have their advantages and should ideally be carried out together. However, in serially repeated samples, karyotyping need not be repeated each time, as the change in percentage of normal and abnormal cells which is better detected by FISH is of greater importance. The cost of the FISH test in our laboratory is half that of karyotyping, and the results of FISH are available in 2 days. A small quantity (1ml) of bone marrow is sufficient for FISH. The FISH test is thus rapidly gaining importance as a useful diagnostic tool in oncology, as more probes are now available for common chromosome abnormalities.

REFERENCES

- Advani SH, Nair R, Bafna A, Gladstone B, Amare (Kadam) P, Saikia T, Parikh PM, Gopal R, Nair CN 1999. Actue promyelocytic leukemia: All trans retinoic acid (ATRA) along with chemotherapy is superior to ATRA alone. *Am J Haematol*, **60**: 87-93.
- Amare (Kadam) PS, Baisane C, Saikia T, Nair R, Gawade H and Advani S 2001. Fluorescence *in situ* hybridization: A highly efficient technique of molecular diagnosis and predication for disease course in patients with myeloid leukemias. *Cancer Genet Cytogenet*, **131**: 125-134.
- Bain BJ 2001. Overview. In: *Best Practice and Research. Clin Haematol*, **14**: 463-477.
- Champlin RE, Golde DW 1985. Chronic myelogenous leukemia: Recent advances. *Blood*, **65**: 1039-1047.
- Cohen N, Rozenfeld-Granot G, Hardan I, Brok-Simon F, Amariglio N, Rechave Gideon, Trakhtenbrot L 2001. Subgroup of patients with Philadelphia-positive chronic myelogenous leukemia characterized by a deletion of 9q proximal to ABL gene: Expression profiling, resistance to interferon therapy and poor prognosis. *Cancer Genet Cytogenet*, **128**: 114-119.
- Druker B, Talpaz M, Resta D et al. 2001a. Efficacy and safety of a specific inhibitor of the Bcr-Abl tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*, **344**: 1031-1037.
- Druker B, Sawyers C, Kantarjan H et al. 2001b. Activity of a specific inhibitor of the Bcr-Abl tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med*, **344**: 1038-1042.
- Heim S, Billstrom R, Kristofferson U, Mandahl N, Strombeck B, Mitelman F 1985. Variant Ph translocations in chronic myeloid leukemia. *Cancer Genet Cytogenet*, **18**: 215-227.
- Huntly BJP, Reid AG, Bench AJ, Campbell LJ, Telford N, Shepherd P, Szer J, Prince HM, Turner P, Grace C, Nacheva EP, Green AR 2001. Deletions of the derivative chromosome 9 occur at the time of the Philadelphia translocation and provide a powerful and independent prognostic indicator in chronic myeloid leukemia. *Blood*, **98**: 1732-1738.
- Kucheria K, Jobanputra V, Sivakumaran TA, Talwar R 1999. Cancer genetics in India. In: *Indo-European Seminar cum Workshop on Advances in Human Cytogenetics*. Lucknow: SGPGIMS. pp. 65-77.
- Madon PF, Athalye AS, Bandkar VM, Dhupal SB, Sopariwala AA, Parikh FR 2002. Cytogenetic analysis of haematological malignancies. In: MK Bhasin, AK Kalla (Eds.): *Expanding Horizons of Human Genetics*, Delhi: University of Delhi.
- Terre C, Bastie JN, Garcia I, Suzan F, Cayuela JM, Therond P, Castaigne S 2001. BCR/ABL fusion gene detected on 9q34 by fluorescence *in situ* hybridization in an acute leukemia with two BCR/ABL positive clones, one Ph-negative and one Ph-positive. *Cancer Genet Cytogenet*, **131**: 37-41.