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Original Article

Diagnostic role of conventional cytogenetics and fluorescence in situ hybridization (fish) in chronic myeloid leukemia patients

Jha CB[1], Kucheria K[2], Choudhary VP[3]
[1]Department of Anatomy, B. P. Koirala Institute of Health Sciences, Dharan, Nepal, [2]Department of Anatomy, [3]Department of Haematology, All India Institute of Medical Sciences, New Delhi, India

Abstract
Introduction: The limitation of cytogenetic analysis is that the Ph chromosome cannot be detected in clumped metaphase or in interphase cells. Fluorescence in situ hybridization (FISH) is a highly sensitive molecular genetic technique, which enables to detect break point cluster region – Abelson (BCR-ABL) complex and minimal residual disease in all Ph positive CML patients not only in metaphase but also in interphase cells.
Aims: To detect Ph chromosome in CML patients by the use of conventional cytogenetics and FISH.
Material and methods: The bone marrow samples were collected in heparinised syringe from 35 diagnosed CML patients and transported to cytogenetic laboratory for chromosomal analysis. Conventional karyotype was prepared by direct harvesting and short-term culture. The FISH analysis was carried out on interphase cells of two patients to confirm the cytogenetic diagnosis.
Results: Out of 35 CML patients, 17 (49.9%) were 100% Philadelphia positive, 10(28.5%) were 50-70% Ph+ mosaics and 3(9%) were 100% Ph negative. In 5 patients (14.25%) cytogenetic analysis failed to confirm the presence or absence of Ph chromosome. FISH was carried out in interphase cells from bone marrow preparations of two patients. The signals for BCR-ABL fusion gene was absent in Ph- negative CML patients. In Ph positive patients, the FISH analysis detected BCR-ABL fusion gene seen as a yellow signal on interphase cells.
Conclusion: Conventional cytogenetics is a useful method for detection of Ph chromosome in metaphase stage of cell division. FISH can be used in interphase stage of cell division for the same purpose.

Key words: CML, FISH, Chromosomal analysis, Philadelphia chromosome

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder resulting from neoplastic transformation of hemopoetic stem cells. Nowell and Hungerford (1960) made the discovery of first chromosomal abnormality in CML patients in a hospital of Philadelphia.[1] The deletion of one of the chromosomes of 22 pair was assumed and this shortened chromosome was named as Philadelphia chromosome in the honour of the city.[2] The Philadelphia (Ph) chromosome, resulting from the reciprocal translocation between chromosome number 9 and 22 t (9; 22)(q34; q11.2) has been identified with advancement of banding techniques in more than 90% cases.[3,4] This translocation became the diagnostic hallmark of chronic myeloid leukaemia. Since then the conventional cytogenetics method is considered as a "gold standard" to determine the percentage of Ph positive (Ph +) metaphases for diagnosis and measuring the disease response to therapy.[5] Most cytogenetic laboratories analyze 20 metaphases selected primarily on the basis of metaphase morphology. This approach is adequate to detect cells with a Ph chromosome. But the Ph chromosome can only be detected at metaphase stage of cell division and is susceptible to sample error and bias toward metaphases with good morphology. These factors can interfere with chromosomal diagnosis to quantify residual disease in response to therapy. It is not always possible to achieve good quality metaphase spreads to detect Ph + chromosome that limited the use of conventional cytogenetics.[6]

Correspondence
Dr. C B Jha
Associate Professor
Department of Anatomy
BPKIHS, Dharan, Nepal
Email: chandraj_2000@yahoo.com

Molecular genetics plays an important role in detection of Breakpoint Cluster Region- Abelson (BCR-ABL) fusion gene in all cases of Ph positive CML patients not only in metaphase but also in interphase cells.[7] FISH is a highly sensitive molecular technique developed parallel with polymerase chain reaction (PCR). This technique enables to detect several nucleic acid target sequences simultaneously with different fluorescent colours.[8] It has been successfully applied for detection of BCR-ABL complex and minimal residual disease in CML patients.

Material and Methods

The bone marrow samples from 35 CML patients diagnosed on the basis of clinical findings and haematological parameters were collected in heparinised syringe by haematologist in the department of haematology and transported to cytogenetic laboratory for chromosomal analysis in All India Institute of Medical Sciences, New Delhi, India. Conventional karyotype was prepared by direct harvesting and short-term culture in RPMI 1640 medium for 24 hours in an incubator at 37^{o} centigrade. GTG banding and photomicrography was done. Well spread metaphases were cut out separately and arranged in pairs according to classification ISCN (1995).[9]

The FISH analysis was carried out on interphase cells of two patients to confirm the cytogenetic diagnosis. For FISH analysis LSI BCR-ABL translocation probe kit (Dual colour probe) and hybrite Denaturation/hybridization (Vysis Germany) were used. The LSI BCR-ABL translocation probe is a mixture of a BCR probe for the BCR locus directly labelled with spectrum green fluorophore and probe for ABL locus, directly labelled with spectrum orange fluorophore. Denaturation of the double strands DNA in the both the probe DNA and the chromosome DNA were done in a solution of formamide at a high temperature (70^{o}). Next the probe was placed on the slide and a glass coverslip was placed on top and sealed with rubber cement to prevent evaporation. The slides were kept in an incubator for overnight for the probe to hybridize with the target cells and bind to it. The strands slowly reanealed. Next day, the coverslip was removed, and the slide was then washed with salt/detergent solution to remove any of the probes that did not bind to the cells. For counter staining (DAPI mountant) was added to specimen area and 22 mm square coverslip was placed on it. The slides were examined and images were captured using smart capture software (Vysis, Germany) and the pictures were printed out.

Results

Cytogenetic analysis was carried out in 35 CML patients. Out of them, 17 (49.9%) were 100% Philadelphia positive, 10 (28.5%) were 50-90% Ph+ mosaics and 3 (9%) were 100% Ph negative. In five patients cytogenetic analysis failed at the time of diagnosis. In patients with Ph+ chromosome the standard Ph translocations (9; 22, t (q34; q11) were observed (Fig.1 and 2). FISH was carried out in interphase cells from bone marrow preparations of two patients. The FISH analysis in Philadelphia negative patient (Lab no301/96) showed two separate red and green signals on chromosome no.9; 22. The BCR-ABL fusion gene was not seen that confirmed the cytogenetic diagnosis of Ph negative CML (Fig.3). In Philadelphia positive patient (Lab.no331/96), the FISH analysis detected fusion of red and green signals (BCR-ABL fusion gene) seen as a yellow signal on interphase cells which proved the cytogenetic diagnosis of Ph positive CML (Fig.4). The normal position of ABL gene is on chromosome no. 9 shown in red colour. The normal position for BCR gene is on chromosome no. 22 shown in green colour. These signals were located on its normal position on chromosome no. 9 and 22 in Ph negative CML patient. In Ph positive patient the ABL red signal juxtaposed with BCR green signal on chromosome no.22 and showed BCR-ABL fusion gene as a yellow signal in between these two signals in interphase cells. This BCR-ABL fusion gene detected by FISH analysis, confirmed the diagnosis of Ph + CML from interphase cells.

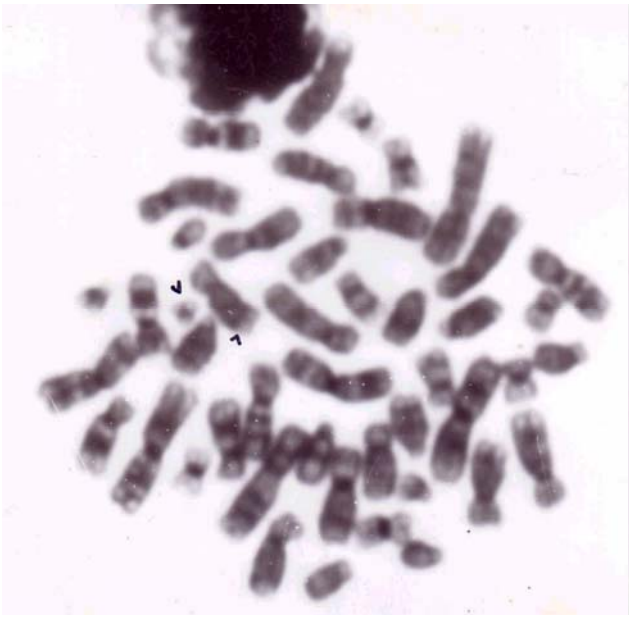

Fig 1: G-banded metaphase spread showing t(9;22)(q34,q11). Arrows indicate 9q+ and 22q-

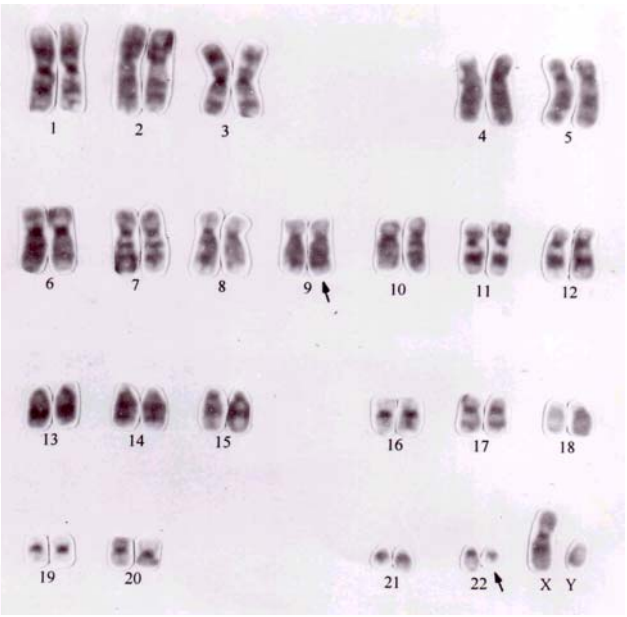

Fig 2: G-banded karyotype showing t (9;22) (q34,q11). Arrows indicate der (9) and der (22)

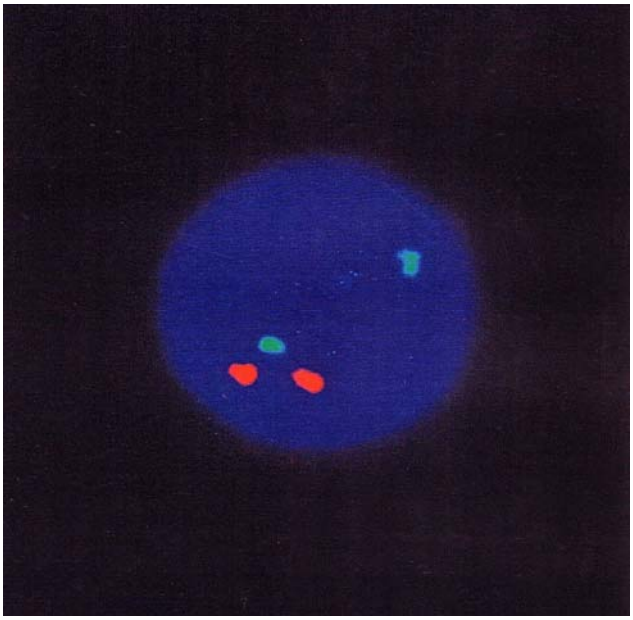

Fig 3: Normal interphase cell of bone marrow from patient 301/96 showing two red (ABL) and two green (BCR) signals.

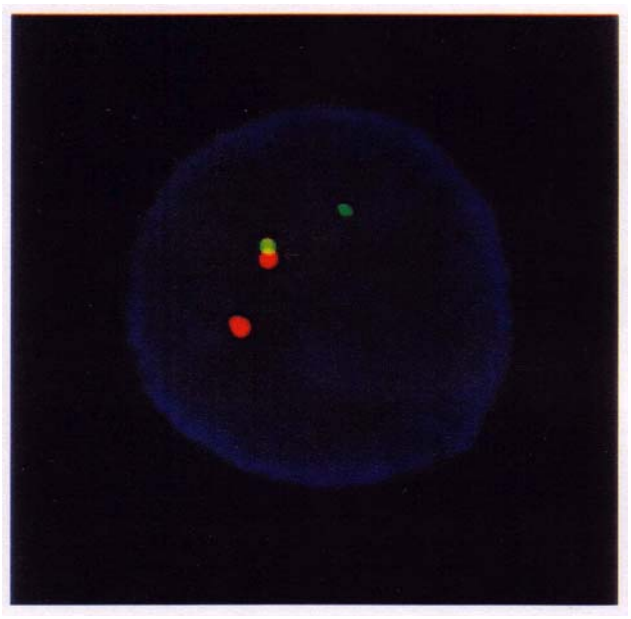

Fig 4: Interphase cell of patient 331/96 showing BCR/ABL fusion as yellow signal, which represents overlapping of BCR (green) and ABL (red) signals. The other red signal represents ABL and green represents BCR.

Discussion

Chronic myeloid leukaemia is associated with a chromosomal abnormality, in which there is a balanced translocation between chromosomes 9 and 22 forming the Ph chromosome.[10] The Ph chromosome is found not only in CML but in 15-30% of adults with ALL, 3-5% of children with ALL, and 1-2% of adults with AML. The presence of the Ph chromosome in acute leukaemia carries a poor prognosis.[11] Conventional cytogenetics is considered as the diagnostic hallmark for identification of Ph positive metaphases in CML. Most of the cytogenetic laboratories analyze 20 metaphases selected on the basis of good chromosome morphology. This approach is adequate to detect cells with a Ph chromosome [8]. In the present study, conventional cytogenetics was done in all cases of CML and 20 metaphase spreads were analyzed. The Philadelphia chromosome (Ph+) was seen in all well spread metaphases in 17 CML patients (Fig.1) and 100% Ph+ CML diagnosis was made in these patients. With the advancement of banding technique Rowley demonstrated that the distal segment of 22 chromosome is translocated to the terminal band in the long arm of chromosome 9.[3] This classical translocation was seen in all Philadelphia positive CML patients (Fig.2). In conventional karyotype of 10 CML patients the 50-70% metaphase spreads showed Ph positive chromosome while in 3 CML patients all the metaphase spreads were Ph negative. It is well known fact that the five to ten percent of CML patients are Ph negative [12] and in the present study 3 patients were suffering from CML but were Ph negative. There were failures of chromosomal analysis in 5 patients. In such patients, chromosomal diagnosis could not be done due perhaps to poor metaphase morphology. The Philadelphia (Ph) chromosome, resulting from the balanced translocation, t (9; 22) (q34; q11.2) generates two fusion genes, BCR–ABL on derivative chromosome 22q, known as the Ph chromosome, and a reciprocal ABL–BCR fusion gene on derivative chromosome 9q. FISH is a new technique, that uses probes to bind to specific sequences at the Ph translocation breakpoints which has been applied extensively at initial diagnosis of CML to investigate cases with failed cytogenetics, to detect BCR–ABL fusion gene, and to decipher complex Ph rearrangements.[13,14] The quantification of BCR-ABL positive cells is achievable through interphase FISH and hence facilitates the monitoring of disease response to therapy[15,16]. In the present situation FISH analysis was done in interphase cells from the bone marrow preparations of two patients to confirm the cytogenetic diagnosis. In Ph negative CML patient (Lab no 301/96) FISH analysis detected two red and two green separate signals for chromosome number 9 and 22 respectively, thereby confirming cytogenetic diagnosis. In Ph + patient (Lab no 331/96) the FISH analysis detected ABL red signal juxtaposed with BCR green signal on chromosome no.22 and showed BCR-ABL fusion gene as a yellow signal in between these two signals in interphase cells. FISH analysis in this patient confirmed the cytogenetic diagnosis of Ph + CML from interphase cells and proved its sensitivity to detect BCR-ABL fusion gene.

Conclusion

Conventional karyotyping is the routine procedure in chronic myeloid leukaemia patients for chromosomal diagnosis from well spread metaphases. It is not always possible to achieve good quality metaphases for chromosomal diagnosis and treatment monitoring. Fluorescence in situ hybridization is a very sensitive technique, which can detect Ph chromosome (BCR-ABL fusion gene), even in interphase cells or poor quality metaphases. FISH should be used in the failed cases of conventional cytogenetic analysis to quantify disease in CML.

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