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Detection of abl/bcr Fusion Gene in Patients Affected by Chronic Myeloid Leukaemia by Dual-Colour Interphase Fluorescence *in situ* Hybridisation

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Abstract

Conventional cytogenetic is the standard technique for detection of Philadelphia (Ph) chromosome in chronic myeloid leukemia (CML). Evaluation of abelson murine leukemia/breakpoint cluster region (abl/bcr) fusion using dual-colour fluorescence in situ hybridization (D-FISH) is an alternative approach allowing rapid and reliable detection of the disease. We employed the technique of interphase D-FISH to detect the abl/bcr fusion in 21 patients and the results were compared to those of conventional cytogenetic analysis. A significant agreement was observed between the results, indicating the accuracy of interphase D-FISH. The technique can overcome the disadvantage of conventional cytogenetic methods, which depends on cell culture and analysis of banded chromosomes, since the analysis is performed by signal counting on uncultured target cells. Furthermore D-FISH provides a more reliable method for evaluating the degree of clone remission to patients with CML after therapy compared to the conventional cytogenetic analysis.

Keywords: CML; Interphase FISH; abl/bcr fusion; Philadelphia chromosome

Introduction

Chronic myeloid leukaemia is the most prevalent myeloproliferative disorder (MPD), with an overall incidence of 2 in 100,000 normal individuals. The disease is characterized by neoplastic overproduction of myelocytes and neutrophils due to malignancy of haematopoietic cells. About 95 percent of patients affected by CML show a derivative chromosome known as Philadelphia (Ph) chromosome, which arises from translocation between long arms of chromosomes 9 and 22 (q34; q11) [1]. The translocation leads to formation of a chimaeric gene coding for a large protein (210 kilo daltons) with a high tyrosine kinase activity [2].

Molecular analysis of DNA from CML patients has revealed that the cellular oncogene for Abelson murine sarcoma virus (abl), which is normally located at 9q34, is translocated to a position within the breakpoint cluster

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region (bcr) at 22q11 [3]. The translocation breakpoint on chromosome 22 is restricted to a small region of 5.8 kb within the bcr gene, between exons 2 and 3 or exons 3 and 4 [4]. In contrast, the translocation breakpoint on chromosome 9 scatters widely within a long sequence at first intron of abl gene (175 kb) [5].

Detection of t(9;22)(q34;q11) is of major importance in diagnosis of CML and monitoring of patient response to therapy. The disease can be diagnosed by routine cytogenetic and banding techniques using bone marrow or peripheral blood samples [6]. However high quality banded chromosomes are not always available and the culture and analysis of the results are time consuming. Interphase fluorescence *in situ* hybridisation (FISH) is a technique that enables rapid detection of chromosomal rearrangements on interphase cells, avoiding the requirement for metaphase cell preparation [7,8]. Several studies have demonstrated the accuracy of interphase FISH in the analysis of bcr-abl rearrangements in CML patients using uncultured lymphocytes and bone marrow cells [9-12]. It has also been shown that there is a definite correlation between results obtained from studies using bone marrow and peripheral blood samples [13-15]. We employed dual colour fluorescence in situ hybridisation and an indirectly labelled probe to study the fusion of abl and bcr genes in patients affected by CML. The results indicate that interphase FISH is a rapid and reliable method for diagnosis and prognosis of CML.

Materials and Methods

Patients

Twenty-one peripheral blood samples (Nine obtained at diagnosis and 12 after treatment) from 21 patients affected with CML and 5 normal peripheral blood specimens were studied during a period of 2 years time. Clinical diagnosis was made by the examination of peripheral blood smear and marrow biopsy. The patients were participants of haematology clinic of Tabriz University of medical sciences. Three to 5 millilitre of heparinised peripheral blood sample was obtained from each patient. The samples were stored at 4°C until used. The storage time was no longer than 3 days.

Lymphocyte Culture and GTG-Banding Assay

Lymphocyte culture was carried out using chromosome elongation methods [16]. The synchronization of cultures was achieved by blocking of DNA synthesis with Thymidine and release of Thymidine block by addition of 2-deoxycytidine to the culture medium. The cells were dropped on to clean microscope slides labelled with serial numbers and stained by GTG-banding methods [17,18]. Twenty-five metaphase spreads were analysed from each culture under a Zeiss axioscope microscope. The karyotypes were prepared using the photographs of the best quality metaphase spreads and the abnormalities were described according to ISCN nomenclature [19].

Molecular Cytogenetics (FISH)

Uncultured lymphocytes were prepared using one millilitre of each sample according to the standard protocols. The interphase cells were fixed on clean microscope slides labelled with the same serial numbers used for cytogenetic method and stored at −20°C until used. Prior to denaturation, the fixed material was examined under a phase contrast microscope and the desired area on each slide was marked. The preparations were then fixed in a fresh fixative (methanol/acetic acid 3:1) for 1 h. The slides were dried and then immersed in acetone for 10 min. After air-drying, the slides were dehydrated through an ethanol series and directly used for denaturation and hybridisation. The target DNA was denatured at 70°C in 70% formamide/2×SSC and dehydrated in cold ethanol series (70%, 90%, 100%).

The commercial probe, used in the present study was a mixture of DNA sequences specific for ABL (on chromosome 9) and BCR (on chromosome 22) genes labelled with biotin-11-dUTP and digoxigenenin-11-dUTP respectively (m-bcr/abl, Minor Breakpoint Translocation DNA Probe, Q-Biogene, P5120-DC). The probe was received as a mixture in hybridisation buffer and was previously denatured. Ten micro litre of probe mixture was incubated at 37°C for 5 min and then applied on a 25X25 mm hybridisation area of each slide containing denatured target DNA. The denatured probe and target DNA were allowed to hybridise at 37°C overnight (12-16 h) under a sealed coverslip.

Post hybridisation washes and simultaneous detection was carried out according to the procedure described in our previous investigation [20] with minor modifications. Following to the hybridisation, the coverslips were floated off by rinsing the slides in 2×SSC at 37°C. The slides were washed twice in 50% formamide/2×SSC at 37°C for 5 min each and then blocked in wash-A solution for 30 min at 37°C. The hybridisation areas were detected as follows: 100 µl of 5 µg/ml FITC conjugated avidin (diluted in wash-A solution) was added for each slide, the area was covered with parafilm and incubated in a 37°C water bath for 15-20 min. Slides were washed three times for 5 min in wash-A at 37°C. 100 µl of a mixture of biotinylated

anti-avidin D (5 µg/ml) and anti-digoxigenin fluorescein Fab fragments prepared in sheep (0.6 µg/ml) diluted in wash A solution was added. The area was again covered with parafilm and incubated for 15-20 min at room temperature. Slides were washed two times for 5 min in wash-A at 37°C and then incubated with 100 µl of a mixture of FITC conjugated avidin (5 µg/ml) and fluorescein anti-sheep IgG (2.25 µg/ml) diluted in wash A for 15-20 min at room temperature. Slides were washed two times for 5 min at 37°C in wash-A and two times for 5 min in 4×SSC, 0.5% Tween-20 at room temperature, dehydrated through an ethanol series (50, 70, 90 and 100 %) and air dried.

The slides were mounted using 10-12 µl DAPI (0.4 µg/ml) in antifade medium AF1 (Citifluor Ltd). The area was covered with a 22×22 mm coverslip, sealed with nail polish and stored at 4°C until used. The results were analysed using an epifluorescence microscope (Zeiss Axioscope) equipped with filter sets specific for DAPI, Propidium iodide, FITC and triple band pass for simultaneous visualisation of red and green signals. For each sample 50-100 hybridised nuclei were analysed. Nuclei without signal, clumped nuclei and nuclei covered with cytoplasm or cellular membrane were not scored. The results were finally compared to those obtained from cytogenetic analysis.

Results

All peripheral blood samples obtained from CML patients were subjected to lymphocyte culture. A minimum of 20 metaphase spreads were analysed for each sample. Four samples failed to produce a result due to poor quality or absence of metaphase cells. Study of banded chromosomes in 13 samples showed Philadelphia chromosome in an average of 95% of the cells (Fig. 1). The remaining 4 samples were revealed to be Philadelphia negative.

The technique of dual colour fluorescence in situ hybridisation (D-FISH) was then employed to localize the abl/bcr probe on its chromosomal location, using normal metaphase spreads. The hybridisation area was simultaneously detected with Rhodamin and FITC and the signals were amplified once. Signal analysis was carried out using a Zeiss Axioscope fluorescence microscope equipped with triple band pass filter set. A distinct red signal was observed on each of 9(q31) indicating for abl and a green signal on each of 22(q11) indicating for bcr on all 50 hybridised metaphase cells which were analysed (Fig. 2).

In order to evaluate the efficiency of probe and hybridisation and detection conditions in interphase nuclei, the probe was initially hybridised to 5 uncultured lymphocyte preparations from normal individuals. A minimum of 50 interphase cells were analysed for presence of expected signals. About 95% of analysed cells showed two red signals specific for abl and two green signals specific for bcr genes (Fig. 3).

The uncultured lymphocytes prepared from CML patients were then hybridised with the probe mixture. A minimum of 100 cells were analysed for each sample. Fifteen cells showed a red, a green and a yellow signal specific for abl, bcr and abl/bcr fusion genes, respectively, in an average of 85.4 percent of hybridised cells (Fig. 4). Four samples showed two red and two green signals on each hybridised cell and no yellow signal indicating for absence of Ph-chromosome. One of the samples exhibited yellow signal in an average of 35% of hybridised cells and the remaining one sample showed yellow signals on 15 out of 200 hybridised cells (7.5%).

Discussion

The present study was performed to evaluate the accuracy of dual colour interphase fluorescence in situ hybridisation in detection of BCR/ABL fusion in patients affected by CML. We used peripheral blood samples to obtain metaphase or interphase cell preparations instead of bone marrow aspirations, since previous investigations had revealed an excellent agreement between results obtained from similar studies using bone marrow and peripheral blood samples [15]. This strategy presents two main advantages; it does not require bone marrow harvesting, and it is more sensitive since much more cells might be analyzed. Moreover, this strategy is cost-effective.

The indirectly labelled probe that we used in interphase FISH studies allowed signal amplification resulted in intensive signals on all hybridisation areas and facilitating the analysis by a conventional fluorescence microscope.

The failure rate of studies carried out by conventional cytogenetic technique was about 19 percent. Two of the four samples, which did not grow in culture medium, belonged to patients who were under-treatment with interferon-alpha, one with hydroxy urea and the remaining one with radiotherapy. This may explain the inhibitory effect of various treatment strategies on growth of the peripheral blood cells. However all interphase cell preparations which were hybridized with probe could produce a result. This indicates for more reliability of interphase FISH experiments in comparison with cytogenetic studies. Furthermore the FISH technique is more rapid and convenient, since there is no requirement for cell culture and the results

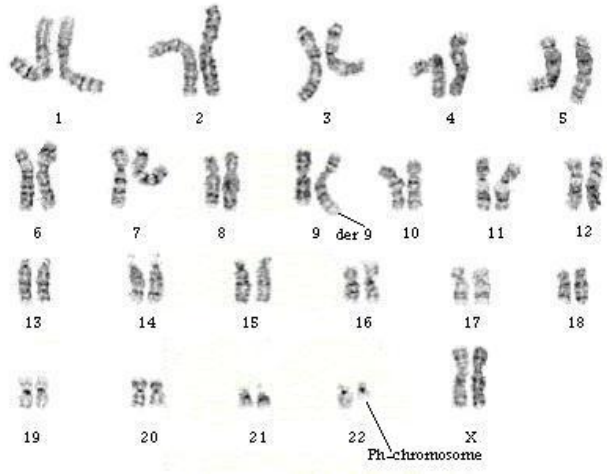

Figure 1. A metaphase spread illustrating Philadelphia chromosome.

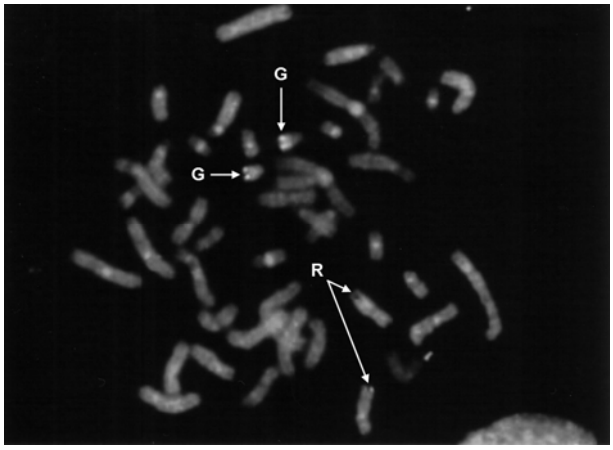

Figure 2. A normal metaphase spread hybridised with probe. The signals detected as R (for red) indicate for ABL gene on chromosome 9 and the signals detected as G (for green) indicate for BCR gene on chromosome 22.

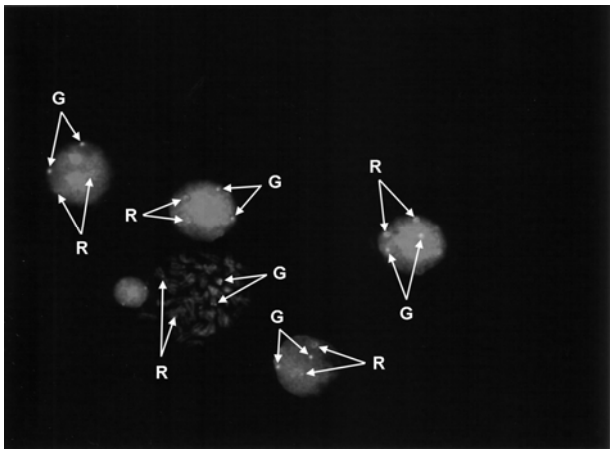

Figure 3. Normal interphase nuclei hybridised with probe. The signals detected as R (for red) indicate for ABL locus on chromosome 9 and the signals detected as G (for green) indicate for BCR locus on chromosome 22.

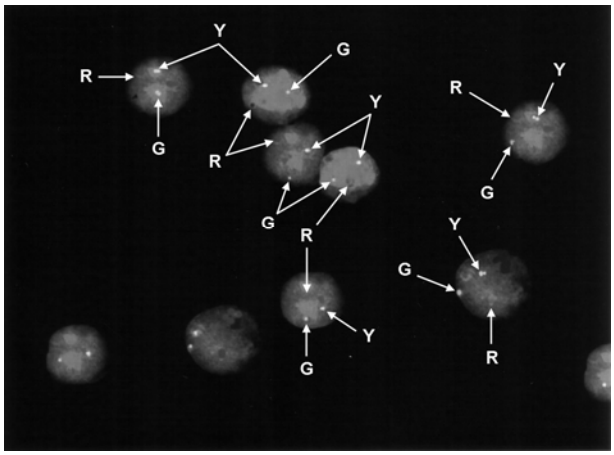

Figure 4. Interphase cells prepared from a CML patient, hybridised with probe. The signal detected as R (for red) indicate for ABL gene, the signal detected as G (for green) indicate for BCR gene and the signal detected as Y (for yellow) indicate for ABL/BCR fusion gene.

are obtained by signal counting instead of analysis of banded chromosomes, which needs highly trained staff.

Within the 15 samples, which demonstrated the signal specific for BCR/ABL fusion gene on an average of 85.4 percent of hybridized cells, 9 samples were related to untreated patients and the 6 others to patients who were under treatment by hydroxy urea. These findings confirm the conventional cytogenetic results and the fact that treatment with hydroxy urea does not affect the Philadelphia chromosome.

Interphase FISH study of samples, which had been revealed to be Philadelphia negative by cytogenetic analysis confirmed the absence of BCR/ABL fusion in two samples, indicating to the complete remission after treatment by Interferon alpha. However a minority of the cells (35% and 7.5%) in the remaining two samples demonstrated the signal specific for BCR/ABL fusion gene. This observation is in agreement with the previous reports that suggested the feasibility of evaluation of response to therapy by interphase FISH using peripheral-blood specimens from CML patients [10,13,14]. However more investigation is required to demonstrate this possibility using the probe set used in the present study.

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