

Real Time Polymerase Chain Reaction in Diagnosis of Chronic Myeloid Leukemia

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ABSTRACT

Objective: To compare the sensitivity and specificity of Real Time Polymerase Chain Reaction (RT-PCR) with conventional cytogenetics in diagnosis of chronic myeloid leukemia.

Study Design: A cross-sectional, analytical study.

Place and Duration of Study: The Armed Forces Institute of Pathology (AFIP), Rawalpindi, from December 2010 to January 2012.

Methodology: A total number of 40 patients were studied, in which all were diagnosed as CML on peripheral blood and bone marrow aspiration. The subjects were tested for the presence of Philadelphia (Ph) chromosome by cytogenetics and BCR-ABL fusion gene by RT-PCR. 2-3 ml of venous blood was collected, half in sodium heparin (anti-coagulant) for cytogenetics and half in EDTA for PCR. For cytogenetics, cells were cultured for 72 hours in RPMI 1640 medium and examined by arresting in metaphase using Colchicine to identify Philadelphia chromosome. For PCR, RNA extraction was done by Tri Reagent LS (MRC, USA) and cDNA was synthesized using reverse transcriptase and gene specific primer. RT-PCR was done on ABI-7500. The positive samples were identified when fluorescence exceeded threshold limit. Results of cytogenetics and RT PCR were compared.

Results: Out of the 40 patients, PCR showed 37 (92.5%) were positive and 3 (7.5%) were negative for BCR-ABL fusion gene, whereas in cytogenetics 28 (70%) were positive for Ph chromosome and 12 (30%) were negative for Ph chromosome. Sensitivity and specificity of cytogenetics was 75.6% and 100% respectively.

Conclusion: Real time PCR as compared to cytogenetics is less tedious, gives quick results, does not require multiple sampling due to culture failure and can be done on peripheral blood.

Key Words: *Chronic myeloid leukemia. Conventional cytogenetics. Real time polymerase chain reaction.*

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm (MPN), characterized by increase proliferation of myeloid cells predominantly granulocytic series in peripheral blood and bone marrow.¹ It is one of the most common haematological malignancies in Asia that involve 15 - 20% of all adult leukemias.² Annual incidence worldwide is 1 - 2 cases per 100,000 populations.³ The natural history of CML involves three phase from chronic phase to an accelerated phase and then finally blast transformation. At presentation, half of the cases have no symptoms and rest present with night sweats, weight loss, left hypochondrial pain and gout. On examination, patients are pale and spleen is enlarged in 50 - 70% of patients.⁴

CML is the most extensively studied malignancy and first malignancy to be associated with specific cytogenetic and molecular abnormality. In 1960, Nowell and Hungerford identified Ph chromosome and its asso-

ciation with CML which was a big step in cancer biology. In 1976, BCR-ABL fusion gene was identified by Jenett Rowley.⁵ This fusion gene is formed due to specific translocation between chromosome 9 and 22. This usually results in the formation of a fusion protein of p 210 kDa oncoprotein which has increase tyrosine kinase activity.⁶ The fusion protein affects multiple cellular processes, which include intracellular signaling, apoptosis, transcriptional regulation and cellular adhesion resulting in a deregulated proliferation. Other than p 210, sometimes p190 and rarely p 230 oncoprotein are also identified.⁷

In the 2008 revision of WHO classification, the term chronic myeloproliferative disorder was replaced by term myeloproliferative neoplasm. According to the World Health Organization (WHO) classification scheme of myeloproliferative neoplasms (MPN) detection of Ph chromosome or BCR-ABL fusion gene confirm the diagnosis of CML and discriminate it from other MPN.^{8,9} Studies have shown that 90-95% cases of CML are Ph chromosome positive, and these cases also have BCR-ABL fusion gene.⁵ In rest 5 - 10% cases of CML Ph chromosome is absent on conventional cytogenetics but these cases usually have complexed translocations and half of them on molecular studies have shown that they have BCR-ABL fusion gene.¹⁰ These cases have similar morphology and clinical course as a typical CML and

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response very well to TKI therapy. Hence, Polymerase Chain Reaction (PCR) can identify complexed translocations undetected by conventional cytogenetics.

These molecular studies have provided important insights into biological activity of BCR-ABL mediated disease pathogenesis. In this new era of molecular study and gene specific diagnosis, pathogenesis and management BCR-ABL gene identification is very vital. In this study, BCR-ABL fusion gene was employed as potential diagnostic modality in the diagnosis of CML as the polymerase chain reaction (PCR) based test is rapid, sensitive and reliable.

The aim of this study was to compare the sensitivity and specificity of Real Time Polymerase Chain Reaction (RT-PCR) with conventional cytogenetics in diagnosis of chronic myeloid leukemia.

METHODOLOGY

This cross-sectional, analytical study was conducted at the Department of Haematology, the Armed Forces Institute of Pathology, Rawalpindi, from December 2010 to January 2012. A total of 40 patients of CML visiting the outpatient reception at AFIP were studied.

After patient reassurance and consent, 2-3 ml of venous blood was drawn from the antecubital vein by aseptic technique. Half was taken in heparin for cytogenetics and half in ethylenediamine tetra-acetic acid (EDTA) for TLC and PCR. Blood counts were performed on Sysmex KX-21 automated haematology analyzer. Total leucocyte count of all individuals was recorded.

For cytogenetics, 0.5 ml of heparinized blood was mixed with 7 ml of RPMI-1640 culture medium in culture vessel and then incubated at 37°C for 72 hours. 0.2 ml of colchicine was added to medium to arrest metaphases and fixed by adding fixative that is 3:1 part absolute methanol/glacial acetic acid. Slides were prepared from cell pellet and stained with Leishman stain. At least 20 metaphases were examined to give Ph positive or negative results.

For RT-PCR, RNA was extracted from peripheral blood leucocytes by using Trizole-LS reagent (USA). Complementary DNA (cDNA) was synthesized by mixing 8 µl of extracted RNA with deoxynucleotide triphosphates (dNTPs), RT buffer, M-MLV enzyme and RNAase inhibitor. A gene specific primer was added to the tube:

ABL-R 5'-GGCCACAAAATCATACGTGCA.

The synthesized cDNA was mixed with PCR mix that contains dNTPs, magnesium chloride etc. along with DNA Taq polymerase and following BCR-ABL primers and probes were used.

BCR3-b3-F 5'-CGTCCACTCAGCCACTGGAT

CR2-b2-F 5'-TGCAGATGCTGACCAACTCG

ABL-a2-R 5'-TCCAACGAGCGGCTTAC

ABL-F 5'-GTCTGAGTGAAGCCGCTCGT

b3a2-b2a2-P 6FAM-CAGTAGCATCTGACTTTGAG
CCTCAGGGTCT-TAMRA

ABL-P VIC-TGGACCCAGTGAAAATGACCCC
AACC-TAMRA

Samples were analyzed by running on ABI-7500 Sequence Detection System (Applied Biosystem). Thermal cycling parameters for PCR consisted of initial 10 minutes at 95°C, followed by 40 biphasic cycles of 15 seconds at 95°C for denaturation and 1 minute at 60°C for annealing and extension. Positive samples were those in which fluorescence exceeds threshold before 35 cycles. Positive and negative controls were run along with test samples.

All the collected data were entered in Statistical Package for Social Sciences (SPSS) version 17. The analyzed variables included numerical data like age and qualitative data like gender. Results for Ph chromosome in cytogenetic analysis and BCR-ABL in PCR were compared with Fisher's Exact test and p-value < 0.05 was taken as level of significance. Sensitivity and specificity were calculated using PCR as gold standard.

RESULTS

A total of 40 subjects were enrolled in the study, all of them were diagnosed as CML on peripheral blood and bone marrow. Out of the 40 CML patients, 26 were males (65%) and 14 were females (35%). Male to female ratio is 1.8:1. The age of patients ranged between 16 - 73 years. Mean age in the CML patients was 37 ± 14 years and mean spleen size was 9.1 ± 5.8 cm. The mean TLC in the CML group was 184.5 × 10⁹/l. Minimum TLC was 17.9 × 10⁹/l and maximum was 552.3 × 10⁹/l. Descriptive statistics were shown in Table I. Results of cytogenetics showed that out of the 40 patients, 28 (70%) were Ph positive whereas 12 (30%) were Ph negative. Out of these 12 Ph negative cases, 4 (10%) patients were given inconclusive result due to inadequate metaphases, in 6 (15%) cases culture failed whereas 2 (5%) cases showed normal karyotype. In PCR, out of the 40 patients 37 (92.5%) were BCR-ABL positive and 3 (7.5%) were BCR-ABL negative. For calculation of sensitivity and specificity 10 inconclusive cases of cytogenetics were included in negative cases. Sensitivity was 75.68% and specificity was 100%. Results of cytogenetics and PCR were shown in Table II. When results of PCR were compared with results of cytogenetics on Fisher's Exact test it was found to be statistically significant with p-value of 0.022.

Table I: Descriptive stats of age, TLC count and spleen.

CML n (40)	Minimum	Maximum	Mean	Standard deviation
Age (years)	16.00	73.00	37.68	14.059
TLC (10 ⁹ /l)	17.90	552.30	184.5025	109.41353
Spleen size (cm)	0	20	9.19	5.896

Table II: Comparative stats of cytogenetics and PCR.

	PCR (gold standard)		Total
	BCR-ABL	BCR-ABL	
	positive +ve	negative -ve	
Cytogenetics			
Philadelphia +ve	28 75.7%(a)	0 .0%(b)	28 70.0%
Philadelphia -ve	9 24.3%(c)	3 100.0%(d)	12 30.0%
Total	37 100.0%	3 100.0%	40 100.0%

Sensitivity = $a/a+c = 28/28+9 \times 100 = 75.68\%$

Specificity = $d/b+d = 3/0+3 \times 100 = 100\%$

p-value = 0.022*

*Fisher's Exact Test was applied to check the significance in the comparison of cytogenetics with PCR (gold standard), $p < 0.05$ was taken as level of significance.

DISCUSSION

Myeloproliferative neoplasms (MPN) are clonal, heterogeneous group of disorders arising from transformation in a haemopoietic stem cell and characterized by proliferation of one or more mature functional cell lines such as granulocytes, platelets or erythroid cells.⁹ All MPN are distinct but closely related disorders. Traditionally MPN have been classified as Philadelphia positive and Philadelphia negative MPN. Philadelphia positive MPN include chronic myeloid leukemia which is defined by its molecular lesion, the BCR-ABL fusion gene, resulting from Philadelphia translocation.¹¹ The discovery of the BCR-ABL fusion gene in CML has further enabled to understand the molecular and cellular basis of this disorder.

CML is a clonal MPN manifested by trilineage myeloproliferation with more dominant proliferation of myeloid series. CML is diagnosed by its characteristic clinical and haematological findings. WHO has formulated guidelines for diagnosis of CML and other MPN, however, there are cases possessing overlapping features. Severance of CML from myeloid hyperplasia due to other primary and secondary haematological disorders has remained to be an intricate job in certain number of cases.¹² Due to differences in clinical picture, disease evolution, prognostic implications and various therapeutic options, the importance of differentiating CML from other causes of myeloid hyperplasia cannot be disregarded.¹³ CML is the commonest adult leukemia in Asia and can be life threatening once progresses to advance phases.² Keeping in view the high sensitivity and significance of this test in establishing the diagnosis of CML, the present study was designed with an aim to evaluate the role of RT-PCR in the diagnosis of CML in a local setting.

The principal observation of this study was the results of cytogenetics and PCR. RT-PCR showed 100% concordance with Ph positive CML cases whereas overall it showed 92% positivity in CML cases. The results of this study are in accordance with the results of local and international studies.

Only few studies have been done in our population due to unavailability of advance diagnostic facilities and in addition cytogenetic studies are quite labour intensive and tedious job. The standard method to study proliferating cells in CML is conventional cytogenetics, however, it is time-consuming and requires high number of viable cells otherwise culture failures occur but in case of PCR mRNA extracted from single cell can give result in a short period of time.¹⁴ Cytogenetics is done on bone marrow aspirate as it contains more dividing cells whereas PCR is done on peripheral blood and would spare the patients discomfort caused by bone marrow biopsy. Cytogenetic analysis has its own limitations in haematological malignancies due to presence of multiple abnormal clones, low mitotic index of disease cell and poor quality of metaphases. To rise above these limitations more sensitive and quick molecular methods like PCR were developed.¹⁵

In a local study, Suhaib *et al.* have established 100% positivity of RT-PCR in 10 untreated Ph positive CML cases in Pakistani population. However, only 10 patients of CML were screened for BCR-ABL fusion gene which is a relatively smaller study group.¹⁶

Anand *et al.* did a similar study in India in year 2012 and had similar results in his study.¹⁷ In 208 CML 200 (96%) were positive for Ph chromosome on cytogenetics whereas BCR-ABL was present in 100% cases.¹⁷

In 1998, Cox *et al.* did a comparative study of cytogenetics and RT-PCR. The sensitivity of RT-PCR related to Ph positive cases was 97%.¹⁸

The results of this study suggest that detection of BCR-ABL fusion gene by RT-PCR is a useful, sensitive and reliable diagnostic test for the diagnosis of CML. Uniformity of PCR results in this study and international studies suggest that PCR is comparable with cytogenetics. In this study, cytogenetics was selected as a comparator as it is most frequently used test in present practice. Addition of PCR testing with cytogenetics increases overall validity of results.¹⁹ Accurate identification of CML is also useful in defining appropriate therapy as BCR-ABL positivity is an absolute requirement of imatinib treatment.²⁰ The ultimate goal of all diagnostic tests is to contribute to improvement in the health of patients. In present era of molecular targeted diagnosis, pathogenesis and therapy it will be very useful to add PCR to other investigations in diagnosis of CML.

CONCLUSION

Real time PCR as compared to cytogenetics is less tedious, sensitive and reliable test. It gives quick and speedy results. It does not require multiple sampling due to culture failure and can be done on peripheral blood. It can be very useful in Ph negative cases with BCR-ABL fusion gene.

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