ONE STEP MULTIPLEX RT-PCR FOR *BCR/ABL* GENE IN MALAY PATIENTS DIAGNOSED AS LEUKAEMIA

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Abstract

Background :
BCR/ABL gene is a product derived from translocation of chromosome 22 to chromosome 9. Its presence confers a diagnostic and prognostic value for Chronic Myeloid Leukemia (CML) and Acute Lymphoblastic Leukaemia (ALL) respectively. The common breakpoints in BCR cluster regions are at b3a2, b2a2 and e1a2 junction.
The main aim of this study was to establish a method in detecting the presence of *BCR/ABL* fusion gene using a One Step Multiplex RT-PCR and to identify types of breakpoints in leukaemia patients in HUSM.

Method :
Twenty-six and 14 Malay patients diagnosed as ALL and CML respectively were enrolled into the study. RNA was extracted from the blood and bone marrow samples and was subjected to One Step Multiplex RT-PCR for *BCR/ABL* gene. The PCR product was visualized directly in ethidium bromide stained gel and photographed. The PCR product for e1a2 breakpoint was 481 bp, 310bp for b2a2 and 385 bp for b3a2. The internal control was the *BCR* gene, which showed an 808 bp PCR product.

Results :
The mean age of ALL and CML patients were 4 years and 43 years respectively. Two of ALL patients (7.1%) had *BCR/ABL* fusion gene, with a breakpoint at e1a2 junction while thirteen of CML (92.8%) patients had the gene. Of these later patients, 85% had a breakpoint at b3a2 junction and 15% at b2a2.

Conclusion
One Step Multiplex RT-PCR has been successfully developed to detect *BCR/ABL* fusion gene. It is a fast and effective technique. The results were comparable with previously reported studies. It should be done routinely in all patients with ALL and CML, as its presence is useful in the management of patients.

Introduction

The Philadelphia chromosome is a shortened of chromosome 22 which is due to a balanced translocation of chromosome 9 and 22. It is the most frequent cytogenetic in human leukaemia. It is detected in more than 95% of patients with chronic myeloid leukaemia (CML), 20% to 40% in adult acute lymphoblastic leukaemia (ALL) and 2% to 5% in paediatric ALL(1)

This t(9;22)(q34;q11) leads to a new fusion gene called the *BCR/ABL* fusion gene. The *BCR* gene on chromosome 9 is juxtaposed next to *ABL* gene located on chromosome 22. There are two common breakpoints in *BCR* gene in leukaemia patients. Most of the breakpoint falls within the major breakpoint cluster region (M-*bcr*), and the resultant *BCR/ABL* mRNA molecules with a b2a2 or b3a2 junction encode a 210-kDa fusion protein. The second breakpoint involves a minor cluster region (m-bcr), which located upstream at e1a2 junction. It is then translated into a 190kDa fusion protein.

In Ph^+ ALL patients the subtype of the protein product can be either 210-kDa or 190-kDa. However 210-kd is hallmark for CML

For many years RT-PCR has been used routinely to detect the presence of BCR/ABL mRNAs. The demonstration of BCR/ABL mRNAs by this technique is a very sensitive assay for the detection of leukaemic clone. However RT-PCR requires multiple steps and primers for the detection of these different breakpoints and it became an expensive and time-consuming test. Thus, multiplex RT-PCR was introduced and in only one reaction three sets of internal and external primers were used for the detection of different PCR products of BCR/ABL mRNAs simultaneously.

To simplify this procedure further, our aim was to establish a one step RT-PCR in which the cDNA synthesis and PCR were performed in a single tube. This step will further reduce the number of manipulation as well as reduced the risk of contamination. Secondly we would like to identify types of breakpoint in the Malay patients diagnosed as leukaemia.

Material and Methods

A total of 40 patients diagnosed as chronic myeloid leukaemia and acute lymphoblastic leukaemia were enrolled in this study. Written consent was taken from them and hospital ethical committee had approved the study.

RNA was extracted from peripheral blood or bone marrow specimen using standard method as in manufacturer protocol (QIAamp®RNA Blood Mini Kits). The presence of RNA was confirmed by running the product on agarose gel.

RT-PCR was performed using RT-PCR kit (Titan™ One Tube RT-PCR kit). The primers for mRNAs transcripts for b2a2, b3a2 and e1a2 were added in the mixture and a total volume of 10μl was achieved in a single tube. Each PCR experiment was repeated at least once and included a negative and three positive controls. A positive K562 DNA control was used to run for every PCR reaction. The other two positive controls were from a known ALL and CML patients with a breakpoint at b2a2 and e1a2 respectively. PCR was performed on a programmable heating block for 35 cycles at 96°C for 1min, 55°C for 50s, 72°C for1min, followed by 10 min extension at 72°C. Reaction products were electrophoresed through a 1.8% agarose gel in a separate room using dedicated pipettes. It was visualized directly in ethidium bromide stained gel and photographed.

Results

A prospective study was done in Hematology Laboratory, HUSM from Jun 2002 to January 2004. During this period, a total of 39 Malay patients newly diagnosed as ALL and 14 as CML out of which nine of them were on follow-up.

Only 26 cases of ALL were included in this study. Thirteen cases were excluded from the study due to inadequate sample and poor quality of RNA.

In ALL, the age of the patients ranged from 14 months to 23 years old with the mean age of 4 years old. There were only two patients (7.1%) with the age above 18 years old. Thirteen patients (46%) were male and 15 patients (54%) were female. Both adult patients were males.

BCR/ABL was detected in two pediatrics female patients diagnosed as ALL. Both exhibited breakpoint at e1a2 junction with PCR product of 481 bp (Figure 1). None had breakpoint at b3a2 or b2a2.

All CML patients were in chronic phase. The mean age was 43 years old. There were 3 patients with the age below 30 years old. Eight (57.1%) of them were male and 6 (42.9%) were female. Only thirteen (92.8%) of them were positive for *BCR-ABL* fusion gene. Of these positive ones, 11 (85%) had a breakpoint at b3a2 junction and 2 (15%) at b2a2 junction (Figure 1). None of them had fusion transcripts at both junctions.

Discussion

In the year 2002, there were 1447 cases of leukaemias reported to the National Cancer Registry, comprising 5.7% of total number of cancers (3). Males to females ratios was 1.4:1 and leukaemias was the fourth commonest cancer in males and fifth in females (2).

In this study a total of 44 Malay patients were newly diagnosed as ALL and CML over a period of two years. Thirty-nine were ALL and 5 were CML. However the test could be performed only on 26 of ALL patients. An additional of 9 patients with CML was included in this study as they were the follow-up cases. These patients were treated either with hydroxyurea or imitinib.

Majority of our ALL patients were in paediatric age group. The BCR/ABL gene was detected in only two (7.1%) of our paediatric patients. Study done by Crist W. noted the presence of *BCR-ABL* gene was usually found in older patients with the median age of 9.6 years old (1). Both of the patients who exhibited positive for *BCR-ABL* in this study were 14 years and 6 years of age respectively. It was expected to have such a low incidence of BCR/ABL gene in our study, as many of our patients were very young with the mean age of 4 years old. This is an important finding as BCR/ABL in children confers an independent bad prognostic factor and an early preparation for stem cell transplant can be made. Both of the fusion gene detected exhibited breakpoints at the e1a2 junction, which is commonly associated in ALL. Later this fusion gene will be translated to p190 k-Da. Children with PH+ ALL have predominantly p190 BCR/ABL expression whereas adult ALL express equally p190 and p210 (3).

In Malaysia, CML constituted 14.6% of total cases of leukaemia, with a slight male preponderance. The median age was 40 years old. Age – adjusted incidence increased with age a similar finding to our study (4).

BCR/ABL gene was found in 92.8 % of patients with clinical diagnosis of CML in our study. One patient was negative for *BCR/ABL* fusion gene. Other studies have found that more than 95% of patients with the diagnosis of CML were positive for *BCR/ABL* fusion gene (5).

The patient who was negative for *BCR/ABL* had clinical features suggestive of chronic myeloproliferative disease. He had translocation (9,22), detected by cytogenetic analysis at the time the diagnosis was made. Patient was treated with interferon for a

year, followed by imitanib myeselate for few months and this molecular study was done after she was started on imatinib. However cytogenetic analysis had not been performed there after. It has been reported in the literature that molecular remission can be achieved in about 3% of patient taking imatinib. The rate of complete and major cytogenetic response are 85% and 79% respectively in patients on imatinib and only 25% in patients taking interferon (6).

Eleven of 13 CML patients (85%) had PCR product of *BCR/ABL* at 385 bp, which corresponded to mRNA at a breakpoint of b3a2 junction encoding for a $p210^{BCR/ABL}$ fusion protein and two patients with PCR product of 310 bp (15%), which corresponded to mRNA product at the breakpoint of b2a2 junction. The similar findings can also be found in other studies (7). However in children with adult type CML, majority of patients expressed b2a2 *BCR/ABL* fusion transcript (8). This was a reversed finding from adult patients which b3a2 was the commonest breakpoint (7).

A number of studies had examined these breakpoints and their influence in the survival or duration of the chronic phase. However the significant of these breakpoints remains controversial (9,10)

RT –PCR has the advantage over cytogenetic and FISH because it can identify both $p190^{BCR/ABL}$ and $p210^{BCR/ABL}$ in one single procedure (11). This method will shorten the time used for diagnosis. However conventional cytogenetic has to complement PCR at initial diagnosis, as additional cytogenetic abnormalities, numerical aberrations and abnormalities other than balanced translocations cannot be detected by later technique. For these reason, multiplex PCR cannot fully replace conventional cytogenetic analysis.

The benefit of multiplex approach is to simplify and shortens the procedure of RT-PCR. In a normal PCR reaction for detection of two different genes, two separate PCR reactions are required and additional amplification of a control gene is also needed to validate the integrity of target mRNA. Therefore the detection of p210 and p190 in single patient require multiple procedures and it is time consuming and expensive. Secondly the amount of RNA used is minimal and we do not need to divide the RNA into multiple reactions. Using multiplex approach a single aliquot of RNA is needed and many different genes could be identified with a single reaction. Multiplex PCR assay is clinically useful, efficient and fast procedure for the detection of genetic changes especially in acute leukemia.

Two stages RT-PCR require cDNA synthesis and PCR in two different tubes and there is a potential risk of contamination. Therefore we had established a single step RT-PCR and the technique is simpler and faster

Conclusion

In this study we have successfully established the technique of one step multiplex RT- PCR for detection of *BCR/ABL* fusion gene in patients with CML and ALL. It is a faster and easy method with comparable results. The technique should be used as a screening test for all patients with ALL and CML.

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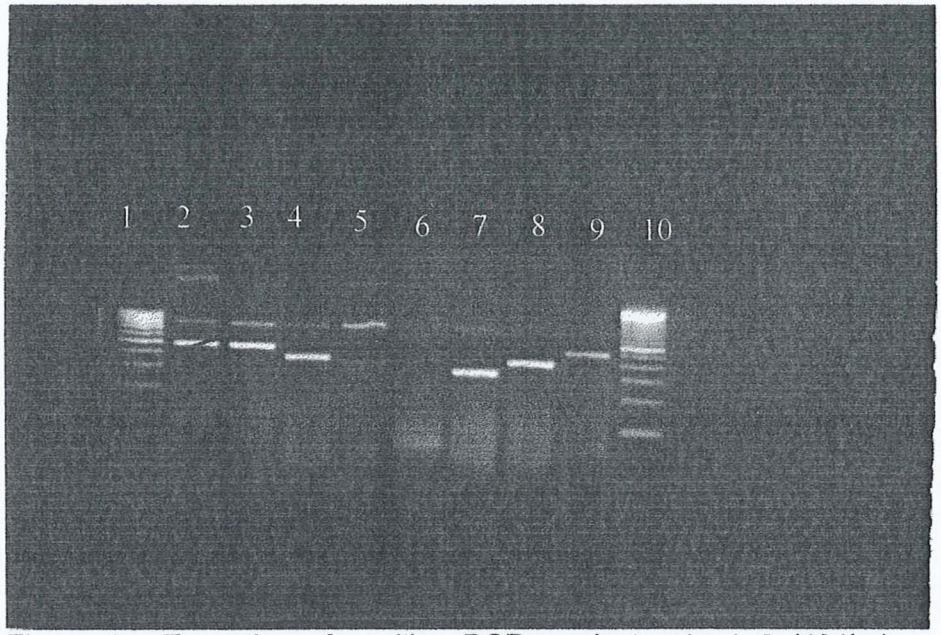

Figure 1: Examples of positive PCR products at e1a2 (481bp) and b3a2 (385bp) breakpoints

Electrophoresis

Lane		Result
1.	100bp ladder	
2.	Patient 1 (ALL)	481bp
3.	Patient 2 (ALL)	481bp
4.	Patient 3 (CML)	385bp
5.	Patient 4 (ALL)	
6	Neg Cont (H2O)	
7.	Control 310 bp	
8	Control 385 bp	
9.	Control 481 bp	
10.	100bp ladder	