Detection of Molecular Variants of BCR-ABL Gene in Bone Marrow and Blood of Patients with Chronic Myeloid Leukemia by Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

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Abstract

Very limited data exists in Thailand regarding the frequency of BCR-ABL leukemic gene and its prognostic implication in Thai CML patients. The objective of this study was to develop a rapid molecular assay for the detection of the two most commonly reported variants of BCR-ABL fusion gene, B2A2 and B3A2 in CML patients. Bone marrow or peripheral blood were used for RNA extraction and reverse-transcribed to cDNA for PCR amplification. 92 per cent of CML patients (91/99) were positive for BCR-ABL gene (61% B3A2 and 31% B2A2). 8/99 CML patients were BCR-ABL-negative. B3A2 and B2A2–positive patients did not have any different clinical and hematological features at presentation although B3A2 patients tended to be slightly older and had higher platelet counts. 71/71 non-CML including other MPD and leukemia cases were all negative for BCR-ABL gene. In conclusion, a rapid RT-PCR assay has now been developed for the detection of this hallmark gene in CML patients. It should be of great value in the differential diagnosis of CML from other diseases. Long-term follow-ups of CML patients with different variants are needed to determine the prognostic importance of each gene variant.

Key word : BCR-ABL Gene Variant, Chronic Myeloid Leukemia

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Chromosomal translocations involved in leukemia have recently been defined at the molecular level[1,2]. In addition to advancing our understanding of the mechanisms underlying the leukemic transformation process, the cloning and sequencing of the genes altered by the translocations have provided new tools for diagnosis and monitoring of patients[3,4]. By routine morphological analysis of

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bone marrow and blood, a small number of leukemic cells in the patients may not be detected. This is in contrast to the recently reported detection ability of PCR amplification method which allows rapid, sensitive and accurate detection of abnormal cells at a very low number[5,6].

The aim of this study was to develop a molecular assay determined to detect the presence of a unique leukemic fusion gene in bone marrow or peripheral blood of patients with CML seen at the Department of Medicine, Siriraj Hospital. This fusion gene arises as a result of balanced translocation between chromosome 9 and 22 (t(9;22)), the Philadelphia (Ph^1) chromosome[7,8]. The Ph^1 chromosome is formed by a translocation that fuses part of the breakpoint cluster region (BCR) gene on chromosome 22 with sequences upstream of the c-abl proto-oncogene (ABL) on chromosome 9 (Fig. 1)[3,4,9]. The functions of these two genes are being characterized worldwide and evidence has suggested their important role as signal transduction intermediates whose aberrant functions may result in abnormal cellular proliferation and growth[10-12]. At present, detection of the Ph^1 chromosome by cytogenetic analysis is cumbersome, expensive and is not routinely available in many centers. In contrast, molecular studies using PCR technology can now be easily performed and they require less time and labor than cytogenetic analysis. In addition, cryptic translocation has been demonstrated whereby BCR-ABL genes are detected at molecular level despite the absence of Ph^1 chromosome by cytogenetic analysis. This research was therefore designed to develop a rapid and specific molecular assay using reverse-transcriptase polymerase chain reaction (RT-PCR) technology to detect the BCR-ABL fusion gene, the frequency of which was also determined in our Thai CML patients. Clinical and laboratory features at diagnosis of CML patients with either gene variant and without the gene were also reviewed.

MATERIAL AND METHOD

Blood or bone marrow samples were collected from adult patients (age > 12 years old) seen at Anantharaj Hematology Clinic, Siriraj Hospital during a routine clinic visit with informed consent. These included 99 CML and 71 other non-CML patients and normal controls. RNA was extracted from blood or bone marrow using Trizol^R and frozen on the same day of sample procurement for further analysis[13]. cDNA was subsequently synthesized from 1 μg RNA using reverse transcriptase enzyme and Superscript II TM Pre-amplification system (Gibco BRL) and used for PCR amplification (RT-PCR) (Perkin-Elmer)[5,14].

PCR assay for CML was established using two different primer sets for the detection of two common BCR-ABL variants, i.e. B3A2 and B2A2 variant. Both variants were the result of chromosomal translocation between chromosome 9 and 22 (Fig. 1 inset)[15]. B3A2 represents the fusion of

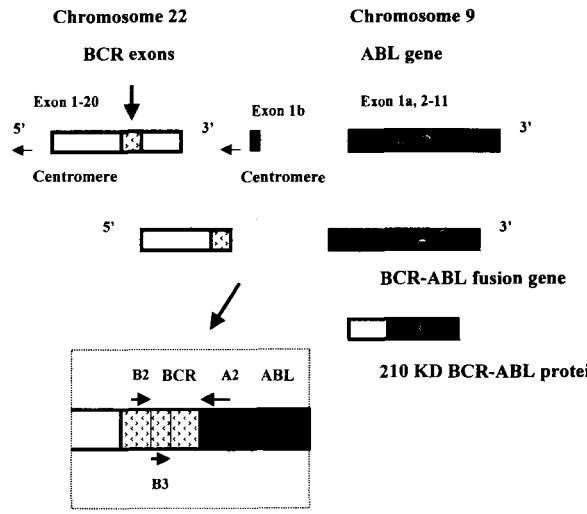

Fig. 1. BCR-ABL fusion gene as a result of translocation between chromosome 9 and 22
The creation of Ph^1 chromosome results in the fusion of the proto-oncogene, ABL gene from chromosome 9 to the novel break-point cluster region (BCR) gene on chromosome 22. The chimeric BCR-ABL gene is transcribed as a hybrid 8.5 kb mRNA and the translated product of 210 kD with tyrosine kinase activity. The translocation breakpoints on chromosome 22 are tightly clustered within the 5.8 kb BCR region whereas the precise breakpoint on chromosome 9 is extremely variable but generally results in translocation of ABL exon 1a or 2 through 11 to relocate adjacent to the BCR exons 2 or 3 on chromosome 22q11.21. The figure in the inset demonstrates the primer sets used to detect the B2A2 or the B3A2 variant of BCR-ABL gene in CML patients.

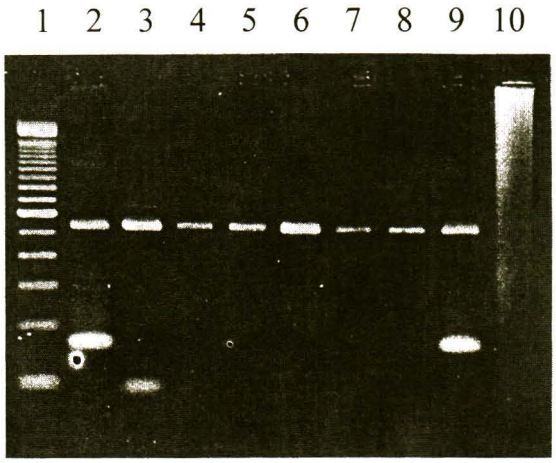

Fig. 2. Results of PCR analysis of representative CML and non-CML patients.
Lane 1 Molecular weight marker
Lane 2 CML-B3A2
Lane 3 CML-B2A2
Lane 4 PV
Lane 5 ET
Lane 6 AML
Lane 7 AMM/IMF
Lane 8 Normal
Lane 9 K562 cell line
Lane 10 Water

BCR exon 3 and ABL exon 2 and gives rise to a 168 base-pair PCR product. B2A2 represents the fusion of BCR exon 2 and ABL exon 2 and gives rise to 93 base-pair PCR product as shown in Fig. 2. Positive control was BCR-ABL-positive K562 leukemic cell line. The sequences of the primers used in this study were as follow:

BCR primer: 5'-CTC CAG ACT GTC CAC AGC ATT CCG-3'

ABL primer: 5'-CAG ACC CTG AGG CTC AAA GTC AGA-3'

Internal control ß-actin sense: 5'-GTG GGG CGC CCC AGG CAC CA-3'

Internal control ß-actin antisense: 5'-GTC CTT AAT GTC ACG CAC GAT TTC-3'(16).

The PCR reactions were performed using the following protocol: 95°C x7 minutes for 1 cycle, 94°C x2 minutes (denaturation), 60°C x2 minutes (annealing) and 72°C x2 minutes (elongation) for 30 cycles and 72°C x10 minutes for 1 cycle. PCR products were subsequently separated in 3 per cent Nusieve (3:1) agarose gel electrophoresis and stained with ethidium bromide for further analysis of the bands.

Clinical and laboratory features of patients were reviewed from available hospital records. Statistical analysis was performed with SPSS 9.0 for window.

RESULTS

Ninety-nine CML samples were screened for BCR-ABL gene by our RT-PCR assay. Sixty patients were positive for B3A2 (61%) and thirty-one patients were positive for B2A2 (31%). Eight patients (8/99) were negative for either variant (8%).

The percentage of positive test in our CML patients was around 92 per cent as shown in Table 1. These patients were defined as CML cases by clinical evaluation, peripheral blood differential counts, bone marrow studies (aspiration or biopsy) and/or low leukocyte alkaline phosphatase (LAP). The major symptoms at presentation included fatigue, weight loss, abdominal discomfort and palpable abdominal mass (Table 2). Very few patients were asymptomatic. Sixty-two per cent of our CML patients were male. Six out of eight BCR-ABL-negative patients were female and fifty-eight out of ninety-one BCR-ABL-positive patients were male (64%). The median and mean age of our CML patients were 36 and 38 years old, respectively.

Table 1. RT-PCR screening in CML and non-CML individuals.

	CML*	Non-CML#
RT-PCR-positive	91	0
RT-PCR-negative	8	71
Total cases	99	71

* peripheral blood and bone marrow aspiration and biopsy confirmed CML disease (in addition to low LAP score in most patients).

\# includes normal individuals (7), lymphoma (5), PV(13) (increased RBC mass, high LAP score), AMM/IMF (11) (BM biopsy + fibrosis), ET/Thrombocytosis (8), CLL (lymphocytosis) (2), atypical MPD (unclassified MPD by BM biopsy in addition to high LAP score (2) and/or atypical features such as marked eosinophilia (3), neutrophilia (3), monocytosis (3) or dysplastic features (1)), anemia of other causes (3), acute leukemia (10).

Table 2. Clinical presentations of Thai CML patients.

	BCR-ABL-Negative	B3A2-Positive	B2A2-Positive
Mean age (yrs)	43	40	35
Age>/ 60 yrs (%)	25	6	3
Sex (M:F ratio)	1:4	1.5:1	2.4:1
Asymptomatic presentation (%)	0	2	5
Weight loss (%)	50	30	35
Palpable mass or abdominal discomfort (%)	75	63	45
Fever (%)	0	10	25
Fatique (%)	38	30	20
Splenomegaly (%)	100	95	100
Spleen >/ umbilicus (%)	71	73	60
Hepatomegaly (%)	75	52	44
Bleeding (%)	12	11	14
Mean WBC count (per mm^3)	151,180	236,923	236,171
	(78,890-240,630)	(47,000-1,000,000)	(40,840-1,120,400)
Mean Hemoglobin (g/dL)	9.30	8.67	9.13
	(6.40-13.00)	(3.50-12.50)	(6.40-14.60)
Mean platelet count (per mm^3)	234,750	662,967	570,115
	(39,000-767,000)	(87,000-2,082,000)	(158,000-2,380,000)

which is younger than the reported age of CML in the literature (45-55). BCR-ABL negative patients were slightly older (mean=43), on average, than BCR-ABL-positive patients. Fifty per cent of CML patients with or without BCR-ABL gene were aged between 21-40. Twenty-five per cent of BCR-ABL-negative and twelve per cent of BCR-ABL-positive patients were more than 60 years old.

The mean white blood cell (WBC) count, hemoglobin (Hb) and platelet counts for our CML patients in this study were 228,524/mm^3, 8.87 g/dL and 592,607/mm^3, respectively. The mean WBC count and Hb was 236,667/mm^3 (40,840-1,120,400) and 8.83 g/dL (3.5-14.60), respectively for BCR-ABL-positive group *versus* 151,180/mm^3 (78,890-240,630/mm^3) and 9.30 g/dL (6.40-13.00), respectively for BCR-ABL-negative group (p=ns). The mean platelet count was 630,778/mm^3 (87,000-2,380,000/mm^3) for BCR-ABL-positive and 234,750/mm^3 (39,000-767,000/mm^3) for BCR-ABL-negative group, respectively ($p<0.05$). A similar proportion of CML patients (70%) with or without BCR-ABL gene had a palpable spleen extending from and beyond the umbilicus. Half of the BCR-ABL-positive group had palpable liver on diagnosis, whereas, 75 per cent of the BCR-ABL-positive group did. All patients who were BCR-ABL-negative had peripheral blood and bone marrow studies compatible with the chronic phase of CML. 1/8 patients had HIV infection discovered at the same time as CML diagnosis and 1/8 had a previous history of spontaneous rupture of the spleen two years before the diagnosis of CML was made.

Sixty of ninety-nine CML cases were RT-PCR positive for B3A2 with 31 of 99 cases positive for B2A2. Clinical characteristics at presentation of both groups are shown in Table 2. The median and mean age, respectively, were 37 and 39.7 years (14-76) for B3A2 and 31 and 35 years (14-70) for B2A2 (p=ns). Male to female ratio was 1.5:1 and 2.4:1 for B3A2 and B2A2, respectively compared to 1:4 in BCR-ABL-negative patients. 70 per cent of B3A2 patients presented with moderate to marked splenomegaly, whereas, 60 per cent of B2A2 patients did. (p=ns). Half of the patients in each group had a palpable liver at diagnosis. The mean WBC count was 236,923/mm^3 (47,000-1,000,000) and 236,171/mm^3 (40,840-1,120,400/mm^3) for B3A2 and B2A2, respectively. The mean Hb was 8.67 g/dL (3.5-12.50) for B3A2 and 9.13 g/dL (6.4-14.6) for B2A2 and the mean platelet count was 662,967 mm^3 (87,000-2,082,000) and 570,115/ mm^3 (158,000-2,380,000) for B3A2 and B2A2, respectively (p=ns). There was no statistical significance with respect to

age, spleen and liver size and WBC count, per cent blast, hemoglobin and platelet counts between the two groups although B3A2 patients tended to be older and had higher platelet counts. Most patients presented in the chronic phase. Two patients with B2A2 transformed into T-ALL, whereas, none of the B3A2 patients did.

Seventy-one non-CML samples were also screened for BCR-ABL gene by the RT-PCR assay (Table 1). These included patients with acute leukemia (10), malignant lymphoma (5), polycythemia vera (13), agnogenic myeloid metaplasia/idiopathic myelofibrosis (11), essential thrombocythemia/ thrombocytosis (8), unclassified myeloproliferative disorders (2), chronic myelomonocytic leukemia (3), chronic neutrophilic leukemia (3), chronic eosinophilic leukemia/hypereosinophilic syndrome (3), chronic lymphocytic leukemia (2), anemia of other causes (4) and seven normal individuals. All non-CML (71/71) cases were negative for BCR-ABL fusion gene.

DISCUSSION

Molecular characterization of abnormal gene(s) in leukemia is currently important and of much interest in western countries. It is useful not only for the diagnosis and classification of types of leukemia but also for the monitoring of the disease after intensive treatment of these patients[5,6].

We have developed in this study a rapid reverse-transcriptase polymerase chain reaction assay for the detection of leukemic gene in Thai patients with CML. Frequency and type of BCR-ABL gene variants in our CML patients were studied. Approximately, 92 per cent of our CML patients were positive for the BCR-ABL gene and none of the non-CML patients or normal individuals were positive, confirming the known specificity of the BCR-ABL gene (particularly, B3A2 and B2A2 variants) for CML patients in the literature[15]. In most series reported in the USA and Europe, approximately 95-98 per cent of CML patients were positive for these two gene variants[15,16]. The other 2-5 per cent of CML patients were reported to be BCR-ABL negative, some of them were classical CML and some were atypical myeloproliferative cases of undefined nature. Atypical CML cases may turn out to be other hematological diseases that need to be further characterized. There is, however, sufficient data in the literature to suggest that BCR-ABL-negative patients have an overall poorer prognosis than the BCR-ABL-positive patients,[15] although the pathophysiology is presently unclear.

The prognosis of our Thai CML patients with respect to BCR-ABL gene variants is currently not known as previous clinical studies of CML in Thailand were performed at the time when molecular technology was not routinely available and therefore the presence or absence of BCR-ABL was not incorporated in the studies[17,18]. Using cytogenetic analysis, Jootar et al found 16 per cent (12/73) of their CML patients at Ramathibodi Hospital to be Ph^1-negative and they appeared to have a worse prognosis than the Ph^1-positive group[17]. It is, therefore, of interest to characterize our patients further into BCR-ABL-positive CML and BCR-ABL-negative CML and determine if the presence of BCR-ABL in our patients confer a better prognosis and overall survival as that reported in the literature[19]. It was of interest to find a slightly higher percentage of BCR-ABL-negative CML patients in our study (8% *vs* 2-5%). Whether it suggests a worse overall prognosis in Thai CML patients is still not known. More patients certainly need to be studied. In our study, BCR-ABL-negative patients were slightly older than the BCR-ABL-positive group with relatively more females in the negative group in contrast to the predominantly male population in the BCR-ABL-positive group. The former group also had lower WBC and platelet counts than the latter. Whether these parameters contribute to a poorer prognosis in the BCR-ABL-negative patients is not known and should be further evaluated in our long-term study of this group of Thai CML patients.

It is of note to mention that rarer variants of BCR-ABL gene, e.g. e19a2, e1a2 will not be detected by our primers. However, those cases usually present with unusual features that will signal the molecular study of the rarer variant[20-22]. For example, e19a2 variant has been reported in chronic neutrophilic leukemia (CNL) whose blood picture is different from the CML cases[22]. We had three CNL patients and all three of them were negative for B3A2 or B2A2 gene variant in our study. In addition, we also had three PCR-negative patients with CMMoL who presented with leukocytosis and monocytosis. These patients belong to the myelodysplastic syndrome and known not to carry the B3A2 or B2A2 variant.

It is of interest to find that two-thirds of Thai CML patients have B3A2 variant which is

similar to the proportion reported in the non-Thai patients although Thai CML patients overall tend to be younger (age 20-40 in contrast to 45-55) and presented at more advanced stages[15,23]. The site of breakpoint in BCR gene has been shown to be clinically important in some studies reported in the literature[24-26]. Precise location of the breakpoint in BCR and ABL and the composition of the fusion BCR-ABL protein have been proposed by several studies to determine the disease phenotype[19]. B3A2 patients have been reported to have higher platelet counts in Japanese studies but not in European and American studies[27,28]. Our study showed the trend toward higher platelet counts in B3A2 patients although it was not statistically significant. The majority of retrospective reports suggest that B2A2 patients do better than B3A2 although recent prospective studies failed to confirm any significant correlation between the breakpoint and disease outcome[29]. Longer follow-up of our patients with either variant is needed to determine its prognostic role in Thai CML patients.

Our study demonstrated that non-CML, e.g, other myeloproliferative disorders (PV, IMF, ET), MDS/CMMoL, CNL, CEoL, acute leukemia, lymphoma as well as normal patients were exclusively negative for BCR-ABL fusion gene confirming the specificity of the BCR-ABL gene for our CML patients. Normal ABL gene is present in normal cells but it produces RNA and protein of different size from the BCR-ABL chimeric gene [30]. Therefore, it will not be detected by our primers that are designed to detect the RNA products of the translocated BCR-ABL gene.

In conclusion, our assay is very useful not only for the initial diagnosis of CML patients but also for the long-term follow-up of CML patients. The prognostic importance of different BCR-ABL gene variants in Thai CML patients will be determined. We are in the process of collecting the follow-up data on clinical and hematological characteristics of CML patients who received either standard chemotherapy or intensified therapy such as stem cell transplantation. The ability to detect the BCR-ABL gene at a low number, i.e. one in 10^5 will certainly help to detect minimal residual disease or early relapse in CML patients whose hematological parameters may still be normal at the time of molecular relapse[5,6]. The importance of molecular relapse will be determined in our future study.

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การตรวจหายีนลิวคีเมีย บีซีอาร์–เอบีแอล จากไขกระดูกและเลือดของผู้ป่วยมะเร็งเม็ดเลือดขาวเรื้อรังชนิดซีเอ็มแอล โดยวิธี อาร์ทีพีซีอาร์

จิรายุ เอื้อวรากุล, พ.บ., ปร.ด., เยาวลักษณ์ อู่ปรัชญา, วท.ม.*, สิริขวัญ บุญเหมาะ, วท.บ.*, สุมิตร วัฒนวิจารณ์, พ.บ. ปร.ด.**

ได้พัฒนาวิธีการตรวจหายีนลิวคีเมีย บีซีอาร์–เอบีแอล ในผู้ป่วยมะเร็งเม็ดเลือดขาวเรื้อรังชนิดซีเอ็มแอล (CML) จำนวน 99 ราย โดยใช้เทคนิค reverse–transcriptase polymerase chain reaction (RT–PCR) อาร์เอ็นเอจากไขกระดูกหรือเลือดของผู้ป่วย ถูกนำมาเปลี่ยนเป็นดีเอ็นเอซึ่งใช้เป็นสารตั้งต้นในการทำพีซีอาร์ กลุ่มเปรียบเทียบได้แก่ คนปกติ และผู้ป่วยโรคเลือดที่ไม่ใช่ CML ผลการศึกษาพบว่า 92% ของผู้ป่วย CML มีความผิดปกติของยีน บีซีอาร์–เอบีแอลในเซลล์จากไขกระดูกหรือเลือด โดยพบว่า 61% เป็น B3A2 variant และ 31% เป็น B2A2 variant ผู้ป่วยทั้ง 2 กลุ่มมีลักษณะคล้ายคลึงกันทางคลินิกเมื่อแรกวินิจฉัย ในกลุ่มที่ไม่ใช่ CML 71ราย พบว่าไม่มีรายใดมียีนบีซีอาร์–เอบีแอลในไขกระดูกหรือเลือด โดยสรุปพบว่าการใช้เทคนิคอาร์ทีพีซีอาร์ในการตรวจหายีนลิวคีเมีย บีซีอาร์–เอบีแอล สามารถทำได้รวดเร็ว ให้ความจำเพาะสูง และมีประโยชน์ในการช่วยวินิจฉัยแยกโรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิด CML จากโรคอื่น ๆ การติดตามผู้ป่วย CML จากการศึกษานี้ในระยะยาวจะช่วยในการบอกการพยากรณ์โรคของผู้ป่วยที่มียีนบีซีอาร์–เอบีแอลทั้งสองกลุ่ม

คำสำคัญ : ยีนบีซีอาร์–เอบีแอล, มะเร็งเม็ดเลือดขาวชนิดซีเอ็มแอล

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