RESEARCH ARTICLE

New Genetic Variation in BCR gene of Major B3a2 Breakpoint BCR-ABL Fusion Gene in Patients with Chronic Myelogenous Leukemia in Yogyakarta, Indonesia

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Abstract

Background: Polymorphic bases in several exons of the BCR gene have been found in several studies of the BCR-ABL fusion gene . Most of the polymorphisms do not have any implications for the primary structure of the BCR-ABL protein. Nucleotide changes are often located in the area close to the fusion region, and therefore may influence primer annealing. Our previous work failed to amplify 15 of 200 samples from BCR-ABL positive chronic myelogenous leukemia (CML) patients using multiplex PCR, the standard method to detect BCR-ABL transcripts used in our institution. The failure was considered due to problems in primer annealing caused by sequence variations. Sequence analysis of BCR-ABL fusion gene breakpoint types in CML patients has never been hitherto performed in Indonesia. Therefore, the aim of this study was to perform sequence analysis of several samples that did not show amplification using the standard method. **Methods:** Fifteen samples were qualitatively amplified by two-step PCR using inner primers in the 2nd PCR to determine the breakpoint type of the BCR-ABL fusion gene. The 2nd PCR products were used as templates to perform sequence analysis, and the results were compared to those in genbank. **Result:** Seven and 5 of 15 samples were confirmed as major b3a2 and major b2a2, respectively. One sample featured a combination of b3a2 and b2a2, and 2 samples a combination of b3a2 and b2a2 with an additional fragment at 500bp. Sequence analysis showed 3 sequence variations in the major b3a2 breakpoint. One had been reported earlier (c.3296T>C) but the others (c.3245C>T and c.3359T>C) were novel. Fragments at 500bp were confirmed as b3a2 and similar sequence b3a2 in genbank. **Conclusion:** This study found two new genetic variations in the BCR gene in BCR-ABL fusion cases.

Keywords: CML- BCR-ABL-b3a2- b2a2- major breakpoint

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Introduction

Chronic myelogenous leukemia (CML) is a hematologic malignancy characterized by excessive accumulation of myeloid cells (Huntly et al., 2003; Perrotti et al., 2010). CML is a relatively frequent disease, corresponding to 15% of total leukemia and 7% to 20% of leukemia in adults (Garcia-Manero et al., 2003; Sillaber et al., 2003). In Asia, the incidence of CML is 0.4-1 per 100.000 individuals per year, and higher incidence has been reported in the Western country, which is 1-2 per 100.000 individuals per year (Garcia-Manero et al., 2003; Baccarani and Dreyling, 2010; Kim et al., 2010 ; Jabbour and Kanterjian, 2014). The median onset of CML patients in Western country is 65 years old. It is 20 years older than the median onset of CML patients in Asia, which is 45 years old (Au et al., 2009; Kim et al., 2010).

Etiophatogenesis of CML mostly involves Breakpoint Cluster Region-Abelson Murine Leukemia (BCR-ABL) fusion gene. The BCR-ABL fusion gene is derived from a reciprocal translocation between chromosome 9 and 22, t(9;22)(q34;q11), known as Philadelphia chromosome. This translocation fuse the 3' part of the ABL gene on chromosome 9 and the 5' part of the BCR gene on chromosome 22, resulting in the formation of the BCR-ABL fusion oncogene (Ohsaka et al., 2002; Sillaber et al., 2003; Druker, 2008). The fusion gene has various sequences. It depends on the breakpoint on BCR gene that fuses with ABL gene. In general, 3 breakpoint cluster regions on BCR gene have been reported, namely major (M-bcr), minor (m-bcr) and micro (μ-bcr) (Smith et al., 2003). Furthermore, several studies has been

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identified polymorphic base of several exons of BCR gene in BCR–ABL fusion gene (De V. Meissner et al., 1998; Saußele et al., 2000). However, most of the polymorphisms do not have any implication to the primary structure of BCR-ABL protein (Saußele et al., 2000).

Standard diagnosis of CML is done based on detection of the BCR-ABL fusion gene include its breakpoint type (Goh et al., 2006). In our institution, the standard methods to detect BCR-ABL fusion gene and its breakpoint type in CML patients are qualitative multiplex RT-PCR and nested PCR. The amplified product is analyzed by using electrophoresis method. However, the method is not sensitive to detect a small genetic variation. The nucleotide changes in the junctional region may influence the annealing of the primer (De V. Meissner et al., 1998).

Between March 2010 and August 2014, 200 blood samples of patients with BCR-ABL positive CML were analyzed in the local laboratory. Fifteen samples could not be amplified by using the method above. The amplification failure was considered as the failure of primer annealing that may be caused by sequence variations. Therefore, this study aims to perform sequence analysis in those 15 samples. Furthermore, sequence analysis of BCR-ABL fusion gene breakpoint types in CML patients was never been reported in Indonesia.

Materials and Methods

Samples and ethical issue

Fifteen cDNA samples were included in this study. The cDNA samples were derived from peripheral blood of BCR-ABL positive CML patients that clinically confirmed at Dr. Sardjito Hospital Yogyakarta Indonesia between March 2010 and August 2014. Patients with other malignancy were excluded. This study was approved by the Ethical Committee of the Faculty of Medicine Universitas Gadjah Mada Yogyakarta Indonesia (No. KE/KF/1029/EC).

Reverse Transcriptase-PCR (RT-PCR)

Two-step PCR by using inner primer in the second PCR was performed in this study. The first PCR was done either using a set of primer (specific conventional PCR) or primers to detect several fragments in one reaction (multiplex PCR). Initially, all of the samples were amplified by using specific conventional and nested PCR. Once the samples could not be amplified using first PCR set, then they were amplified using multiplex and nested PCR. Figure 1 showed the algorithm of BCR-ABL fusion gene amplification in this study. Conventional or multiplex reverse trancriptase (RT)-PCR was performed according to previous study (Goh et al., 2006). RT-PCR was performed by using Biorad C1000 thermocycler machine and PCR kit from Roche (cat.#04 738 314 001). K562 cells line carrying b3a2 major breakpoint type was used as positive control. All PCR results were visualized by electrophoresis method using 2% agarose gel.

Table 1 and 2 demonstrated the primers to amplify breakpoints of interest. The product of conventional PCR can be seen at 616bp for b3a2 and 552bp for b2a2 and for nested PCR should be at 443bp for b3a2 and 368bp for b2a2. The product for multiplex PCR had to be at 627bp for b3a2; 552bp for b2a2; 429bp for minor e1a2; and 1,167 bp for micro e19a2. The expected band size for micro breakpoint type (e19a2) (sequences on exon 19 of BCR gene and exon 3 of ABL gene) was 206bp for e19a2.

Sequence analysis

All of PCR products were sequenced. The sequencing results were analyzed by using MEGA 5.1 software. The sequencing results were aligned to BCR-ABL sequence from genbank according to their breakpoint type.

Results

Conventional RT-PCR was used to detect a specific rearrangement of BCR-ABL transcript. All samples (n=15) amplified by conventional RT-PCR using specific primer for major breakpoint type showed negative results (Figure 2A). All products of the conventional RT-PCR were used as DNA template for the second nested PCR using inner primer. Thirteen of 15 samples showed positive band and 2 samples (no 36 and 140) were negative. Seven samples (no 28, 35, 38, 132, 165, 170 and 185) showed band at 443 bp, which were in similar size with positive control band of K562 cell line. Thus, those samples were confirmed as b3a2 major breakpoint. Two samples (no 137 and 138) had fragment at 368bp, specific band for b2a2 major breakpoint. Four samples (no 147, 158, 162, and 164) showed band at 368bp size and additional band (Figure 2B). In order to clarify the position of the bands of samples no 147, 158, 162, and 164, electrophoresis was

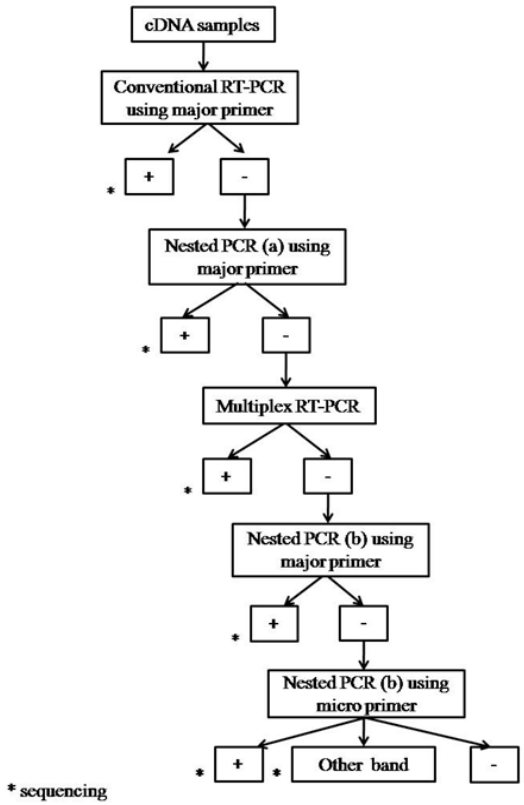

Figure 1. Algorythm of PCR for BCR-ABL Transcript Type Identification

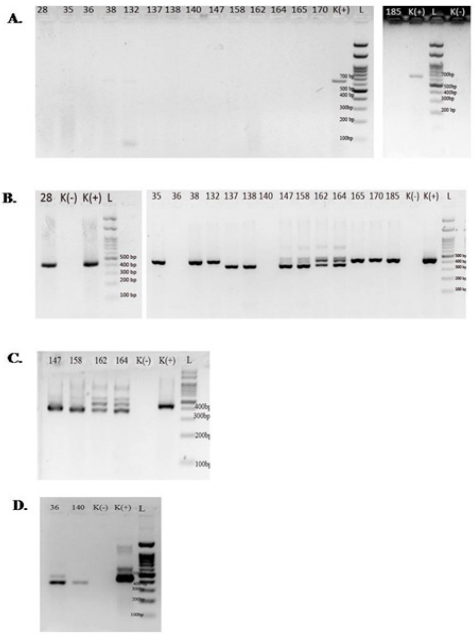

Figure 2. Electrophoresis Result of Every Stage of PCR. A. Conventional RT-PCR with major primer. All samples showed negative result; B. Nested PCR (a) with major primer. Samples 28, 35, 38, 132, 165, 170 and 185 were confirmed as b3a2 major breakpoint type; Samples 137 and 138 were confirmed as b2a2 major breakpoint type; Samples 147, 158, 162, and 164 were confirmed as b2a2 major breakpoint type with additional band; C. Electrophoresis on 4% agarose gel. Samples 147 and 158 were confirmed as b2a2 major breakpoint type; Samples 162 and 164 were confirmed as a combination of both b3a2 and b2a2 type with additional band at 500 bp size; D. Nested PCR (b) using major primer. Sample 36 was confirmed as a combination of both b3a2 and b2a2 type; Sample 140 was confirmed as b2a2 major breakpoint type: (L) DNA ladder; (K+) positive control from K562 cell line; (K-) negative control.

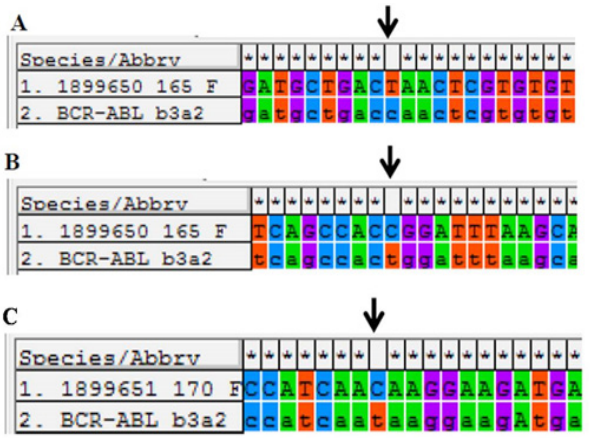

Figure 3. Sequence Analysis Results. A. BCR exon 13 of Sample 165. Arrow shows sequence variation at 47th base, C replaced by T; B. BCR exon 14 of Sample 165. Arrow shows sequence variation at 56th base, T replaced by C. C. BCR exon 13 of Sample 170. Arrow shows sequence variation at 98th base, T replaced by C.

repeated using 4% agarose gel. Samples no 147 and 158 confirmed as b2a2 major breakpoint. Samples162 and 164 had combination of both b3a2 and b2a2 breakpoint with additional band at around 500bp (Figure 2C). Samples no 36 and 140, which gave negative result in conventional and nested PCR were amplified by using multiplex RT-PCR and nested. Nested PCR using primer specific for major breakpoint confirmed that Sample 36 had a combination of b2a2 and b3a2 breakpoint; and Sample 140 had major b2a2 breakpoint type (figure 2D). Results of the RT-PCR are listed in Table 3.

All samples were sequenced, and the nucleotides sequence was compared to the ones from genbank as the standard. Sequence analysis found that four samples had b3a2 breakpoint type (BCR-ABL-28, -35, -38, -132, and -185) and five samples had b2a2 major breakpoint type (BCR-ABL-137, -138, -140, -147, and -158) and the sequences were similar to the ones in genbank for each type. Sample no 36, which was confirmed as a combination of b3a2 and b2a2 type, had similar sequences with b3a2 and b2a2 in genbank. Sequences of both b3a2 and b2a2 types in samples number 162 and 164 were also similar to the ones in genbank. Additional band at

Table 1. Primer Sequences of Major Breakpoint in Conventional RT-PCR and Nested PCR (Goh et al., 2006).

Primer	Conventioanl RT-PCR	Nested PCR nested (a)
Sense	5'-ctacggagaggctgaagaa-3' (BCR exon 11)	5'-gtgcagagtggagggagaac-3' (BCR exon 12)
Anti sense	5'-cgtgatgtagttgcttggga-3' (ABL exon 3)	5'-acaccattccccattgtgat-3' (ABL exon 3)

Table 2. Primer Sequences in Multiplex RT-PCR and Nested PCR (Goh et al., 2006).

Primer	Multiplex RT-PCR	Nested PCR (b)
Sense	5'-gctacggagaggctgaagaa-3' (BCR exon 11) 5'caacagtccttcgacagcag-3' (BCR exon 1)	Major : 5'-gtgcagagtggagggagaac-3' (BCR exon 12) Micro : 5'-cttcgacgtcaaagcccttc-3' (BCR exon 19)
Antisense	5'-cgtgatgtagttgcttggga-3' (ABL exon 3)	Major : 5'-acaccattccccattgtgat-3' (ABL exon 3) Micro : 5'-ctaagacccggagcttttca-3' (ABL exon 3)

Table 3. Breakpoint Type of CML Patients' Samples

No	Sample Code	Breakpoint Type
1	BCR-ABL-28	B3a2
2	BCR-ABL-35	B3a2
3	BCR-ABL-36	B3a2 and b2a2
4	BCR-ABL-38	B3a2
5	BCR-ABL-132	B3a2
6	BCR-ABL-137	B2a2
7	BCR-ABL-138	B2a2
8	BCR-ABL-140	B2a2
9	BCR-ABL-147	B2a2
10	BCR-ABL-158	B2a2
11	BCR-ABL-162	B3a2, b2a2 and 500 bp
12	BCR-ABL-164	B3a2, b2a2 and 500 bp
13	BCR-ABL-165	B3a2
14	BCR-ABL-170	B3a2
15	BCR-ABL-185	B3a2

500bp in two samples showed similar sequence with b3a2 breakpoint in genbank.

Two samples with b3a2 major breakpoint type (Samples 165 and 170) showed sequences variations. Two sequence variations were found in Sample 165, which are at 47th base of exon 13 and 56th base of exon 14. Both are in BCR gene. The 47th base of exon 13 BCR gene (Cytosine) was replaced by Thymin, c.3245C>T (Genbank accession NM_021574.2; nomenclature according to Antonarakis, 1998) (Figure 3A). This change was occurred at third nucleotide of the codon, and led to silent mutation. The codon changed from ACC into ACT without any amino acid change, which is Threonin. The second sequence variation was the replacement of Thymine into Cytosine at 56th base of exon 14 BCR gene, c.3359T>C (Genbank accession NM_021574.2; nomenclature according to Antonarakis, 1998), corresponding to ACT and ACC (Figure 3B). The change also led to silent mutation, because both codons encode Threonin. Those two sequence variations in Sample 165 had not been reported yet. Further study need to be done to determine whether these new variations are polymorphism in the BCR gene.

Sequence variation of Sample 170 was the replacement of Thymine into Cytosine at 98th base of exon 13 BCR gene, c.3296T>C (Genbank accession NM_021574.2; nomenclature according to Antonarakis, 1998). This change was also occurred in the third nucleotide of the codon, AAT changed into AAC. Both codons encode Asparagine. Sequence variation of sample 170 had been reported as polymorphism in previous studies by De V. Meissner et al., (1998) and Saußele et al., (2000) (Figure 3C). The results of sequence analysis are listed in Table 4.

Table 4. Sequence Analysis Results

Sample Code	Fragment size (bp)	BCR exon 12	BCR exon 13	BCR exon 14	ABL exon 1	ABL exon 2	ABL exon 3	Additional Sequence / sequence variation	Conclusion
28	443	+	+	+	-	+	+	-	B3a2
35	443	+	+	+	-	+	+	-	B3a2
36	368	+	+	-	-	+	+	-	B2a2
	443	+	+	+	-	+	+	-	B3a2
38	443	+	+	+	-	+	+	-	B3a2
132	443	+	+	+	-	+	+	-	B3a2
137	368	+	+	-	-	+	+	-	B2a2
138	368	+	+	-	-	+	+	-	B2a2
140	368	+	+	-	-	+	+	-	B2a2
147	368	+	+	-	-	+	+	-	B2a2
158	368	+	+	-	-	+	+	-	B2a2
162	368	+	+	-	-	+	+	-	B2a2
	443	+	+	+	-	+	+	-	B3a2
	500	+	+	+	-	+	+	-	B3a2
164	368	+	+	-	-	+	+	-	B2a2
	443	+	+	+	-	+	+	-	B3a2
	500	+	+	+	-	+	+	-	B3a2
165	443	+	+	+	-	+	+	47th base of BCR exon 13, C→ T;	B3a2; c.3245C>T
								56th base of BCR exon 14, T→C	B3a2; c.3359T>C
170	443	+	+	+	-	+	+	98th base of BCR exon 13, T→C	B3a2; c.3296T>C
185	443	+	+	+	-	+	+	-	B3a2

Discussion

Molecular method in CML diagnosis in Molecular Biology Laboratory, Faculty of Medicine, Universitas Gadjah Mada (UGM)-Dr. Sardjito hospital has been done since 2010. The qualitative method used in this study was two-step PCR by using inner primer in the second PCR (nested). The two-step PCR included either conventional RT-PCR and nested PCR or multiplex RT-PCR and nested PCR. The methods used in this study were modified from the ones previously reported (Goh et al., 2006).

Since more than 95% of CML patients had major breakpoint type (Pane et al., 2002; Shet et al., 2002; Goh et al., 2006), the initial amplification used in this study is conventional RT-PCR to amplify the major breakpoint. All samples (15 samples) showed negative results. They were further amplified by using nested PCR using inner primer for major breakpoint. Nested PCR was a variant of PCR using a pair of primer annealling to the inside area of the first PCR product. Therefore, the product of nested PCR was shorter than the first PCR products. Nested-PCR was a very sensitive and specific method. The sensitivity of nested-PCR was two to three times higher than the first step PCR (Peres de Rozas et al., 2008).

The amplification by nested PCR for major breakpoint found that 13 samples were confirmed as major breakpoint, while the other two were negative. The two negative samples were further confirmed that one sample having a combination of b3a2 and b2a2 major breakpoints and the other one have b2a2 major breakpoint. The samples were success to be amplified after the modification of the cycles from 40 to 30 (data not shown). One of the factors that might influence the amplification failure was cycle number. Too many PCR cycles might increase nonspecific amplification (Roux, 2009).

Overall, the result got 20 fragments from RT-PCR, that is 7 samples of major b3a2, 5 samples of major b2a2, 1 sample had a combination of b3a2 and b2a2 types and 2 samples had a combination of b3a2 and b2a2 type with additional fragment at 500bp. All of 20 fragments were sequenced. Sequence analysis showed that all of the fragments with b2a2 type had similar sequences with the b2a2 sequence in genbank. Eight of 10 fragments with b3a2 type had a similar sequence with b3a2 sequence in genbank, while the other two (sample no 165 and 170) had sequence variations.

Two new sequence variations in sample no 165 (c.3245C>T and c.3359T>C) never been reported yet, while the other one found in sample no 170 (c.3296T>C) had been reported as a polymorphism in previous studies by De V. Meissner et al.(1998) and Saußele et al. (2000). All of the variations (mutations) found in this study did not cause the change of amino acid. Therefore, they do not have implication in the structure of BCR-ABL protein (Saußele et al., 2000). However, eventhough silent mutationt supposed to be have no effect, several studies showed that silent mutation gave harmful effects, by speeding up or slowing down protein synthesis, or affecting the splicing (Mynampati et al., 2016; Yadegari et al., 2016). In this study, we did not see hematological and clinical data of the patients with new mutations, thus further study need to be done to see whether those "silent mutation" have any correlation with disease status of the patients. In this study, the mutation was found in only one patient. Study in the population that represent the case is need to prove whether the mutation have harmfull effect to the disease. In addition, the use of samples derived from bone marrow is important because the origin of the disease in the bone marrow, and in this case is to know whether the mutation is germline or somatic.

Other than functional effects, silent mutation showed in the sample 170 was identified in the 8th position before the junctional region of BCR-ABL cDNA (De V. Meissner et al., 1998). Due to the location of alteration was close to the fusion region, it might have a significant influence on the annealing of PCR primers, probes for real time PCR, and antisense oligonucleotides. A thymidine/cytosine replacement occurs frequently in BCR exon b2. In order to develop the real time PCR for BCR-ABL transcript, the designed probe should not be at the critical region to avoid underestimation of the number of BCR- ABL transcripts (Saußele et al., 2000).

The sequence of additional bands at 500bp size in sample number 162 and 164 were similar to the b3a2 sequence in genbank. Thus, the additional bands were suspected as an unspecific band that could be caused by several conditions, such as too many PCR cycles, too long annealing time, too low annealing temperature, and too long extension time. PCR components could also be the source of the problem, such as too high concentration of primers, dNTPmix or water contained impurities, or too high concentration of Mg2+ (Henegariu et al., 1997; Roux, 2009).

In conclusion, this study found two new base variations in BCR gene of BCR-ABL fusion gene, namely c.3245C>T in exon 13 and c.3359T>C in exon 14. Both variations were silent mutations. Further study need to be done to explore whether the mutations gave harm effect to the disease.

Statement of conflict of Interest

The authors declare that they have no conflict of interest.

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