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INDONESIAN JOURNAL OF CLINICAL PATHOLOGY AND MEDICAL LABORATORY

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RESEARCH

COMPARATIVE RATIO OF BCR-ABL GENES WITH PCR METHOD USING THE CODIFICATION OF G6PD AND ABL GENES IN CHRONIC MYELOID LEUKEMIA PATIENTS

(Perbandingan Angka Banding Gen BCR-ABL Metode PCR Menggunakan Baku Gen Glucosa-6-Phosphate Dehidrogenase dan Gen Abelson Kinase di Pasien Chronic Myeloid Leukemia)

Tonggo Gerdina Panjaitan¹, Delita Prihatni¹, Agnes Rengga Indrati¹, Amaylia Oehadian²

ABSTRAK

Chronic Myeloid Leukemia (CML) adalah keganasan hematopoetik pertama yang dihubungkan dengan jejas genetik. Chronic Myeloid Leukemia digolongkan sebagai penyakit mieloproliferasif kronis disebabkan translokasi resiprokal kromosom 9 dan 22 yang disebut kromosom Philadelphia (Ph). Kromosom Ph membentuk gen yang disebut BCR-ABL. Pemeriksaan molekuler CML bertujuan untuk mengetahui aktivitas transkripsi mRNA gen BCR-ABL, yang berguna untuk menetapkan diagnosis dan pemantauan pengobatan pasien CML. Saat ini, WHO mempublikasikan ada sembilan (9) gen baku yang digunakan secara luas. Tujuan penelitian ini adalah untuk mengetahui angka banding gen BCR-ABL/G6PD dan BCR-ABL/ABL di pasien CML dengan kromosom Ph (+) secara membandingkan. Penelitian ini menggunakan 79 bahan biologis tersimpan (BBT) mRNA Ph (+) dari leukosit pasien CML yang datang ke RSUP Dr. Hasan Sadikin Bandung selama masa waktu antara bulan April 2012–April 2014. Pemeriksaan angka banding gen BCR-ABL/G6PD dengan metode Real-time Quantification PCR menggunakan alat LightCycler® Roche. Angka banding gen BCR-ABL/ABL diperiksa menggunakan alat Bioneer®. Gen baku G6PD dapat mendeteksi tipe b2a2, b3a2 dan e1a2. Gen baku ABL hanya dapat mendeteksi tipe b2a2 dan b3a2, tetapi lebih stabil bila dibandingkan dengan gen baku G6PD. Bentuk penelitian adalah perbandingan analitik dengan rancangan kajian potong lintang. Analisis statistik menggunakan uji nonparametrik Wilcoxon. Hasil angka banding mRNA gen BCR-ABL/G6PD dan gen BCR-ABL/ABL [1,93% (0,0–59,7 fg) vs 15,37% (0,04–35,7 kopi), $p < 0,001$]. Gen BCR-ABL tidak terdeteksi di 3 BBT dengan menggunakan gen baku ABL. Berdasarkan penelitian ini, dapat disimpulkan, bahwa terdapat perbedaan bermakna antara angka banding gen BCR-ABL/G6PD dan yang terkait BCR-ABL/ABL. Gen baku yang sama diperlukan untuk mendiagnosis dan memantau respons pengobatan.

Kata kunci: CML, gen BCR-ABL, RQ-PCR, G6PD, ABL

ABSTRACT

Chronic myeloid leukemia was the first malignant disease of hematopoietic that had the relation to genetic lesion. Chronic myeloid leukemia characterized as chronic myeloproliferative disorder, caused by reciprocal translocation of chromosome 9 and 22 which also called the Philadelphia chromosome. Philadelphia chromosome gene form is also called BCR-ABL. Molecular assay in CML is to know mRNA BCR-ABL gene transcript activity that used for the diagnostic and therapy monitoring of CML patients. Recently, the World Health Organization had published nine standard genes which widely used. The aim of this study is to know the ratio of BCR-ABL/G6PD gene and BCR-ABL/ABL gene in Philadelphia chromosome by comparing. This study included 79 archived biological materials from leukocyte inhabited by CML patients Ph (+) who came to RSUP Dr. Hasan Sadikin Hospital Bandung, which obtained from April 2012–April 2014. The ratio of BCR-ABL/G6PD gene have been evaluated with Real-time Quantification PCR (LightCycler®) Roche. By using G6PD standard gene could detect b2a2, b3a2, and e1a2 type. The BCR-ABL/ABL gene ratio has been evaluated using (Bioneer®) equipment. Abelson standard gene could only detect the b2a2, b3a2 type, which is more stable than the G6PD gene. The research carried out is cross sectional study with comparative analytic design. The statistical analysis was performed using Wilcoxon

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non-parametric test. The ratio of mRNA BCR-ABL/G6PD gene and BCR-ABL/ABL gene: [1.93% (0.0–59.7 fg) vs 15.37% (0.04–35.7 copies), $p < 0.001$]. The BCR-ABL gene was undetectable in three (3) samples using ABL gene standard. Based on this study, it can be concluded that there is significant difference between BCR-ABL/G6PD gene ratio and BCR-ABL/ABL gene one. The same standard gene should be used in subsequent BCR-ABL gene ratio assay for diagnosing and treatment response monitoring.

Key words: CML, BCR-ABL gene, RQ-PCR, G6PD, ABL

INTRODUCTION

Chronic Myeloid Leukemia (CML) is the first hematopoietic malignancy associated with typical genetic lesion, and classified as a chronic myeloproliferative disease. The condition is caused by a reciprocal translocation of chromosomes 9 and 22, known as *Philadelphia* chromosomes.^{1,2} This translocation forms BCR-ABL fusion proto-oncogenes strongly suspected as the main cause of CML.^{3,4} *Philadelphia* chromosome (+) is found in 95% of patients with CML, while *Philadelphia* chromosome (–) usually has a poor healing forecasts. CML incidence is between 1–2 cases per 100,000 people per year with a ratio of 2:1 between male and female patients. *Chronic myeloid leukemia* can also be considered as leukemia commonly found in middle age, mostly between the ages of 30–50 years, but it can also occur in children, known as *Juvenile CML*.¹

In Indonesia, CML is the most common leukemia, about 15–20% of all leukemia cases.⁵ Data from the Ministry of Health in 2012 show that the number of cancer patients in Indonesia reached 430/100,000 person.⁶ Medical record data in Dr. Hasan Sadikin Hospital in 2013 also show that there were 39 of 196 leukemia patients hospitalized suffering from CML, consisted of 25 male patients and 14 female patients, five of whom died. Other data from Biomolecular Laboratory of Clinical Pathology Department of Dr. Hasan Sadikin Hospital show that as many as 174 patients diagnosed with CML had been examined for their molecular genes, BCR-ABL, since April 2012 to April 2014.⁷

Thus, this research aimed to determine ratio of BCR-ABL genes with RQ-PCR method using G6PD and ABL genes in CML patients hospitalized in Dr. Hasan Sadikin Hospital. The significance of this research is to help the diagnosing and monitoring of the treatment progress in patients with CML. This research is also expected to provide an explanation regarding the comparative ratio of BCR-ABL genes with RQ-PCR method using the codification of G6PD and ABL genes for working practice in laboratory.

Chronic myeloid leukemia is the first hematological malignancies associated with typical genetic lesion.^{1,2} These typical genes found in CML were first identified in 1845, and then in 1960 was called as *Philadelphia chromosome* (Ph), identified by the Medical Faculty of Pennsylvania in Philadelphia, by Nowell and Hungerfort.^{1,3,8} In general, this disease is a disorder of clonal stem caused by exposure to radiation, cytotoxic drugs, and chemicals like benzene. However, factors causing over 95% of CML cases are still unknown. *Chronic myelogenous leukemia* is not a hereditary disease, but an acquired disease.^{1,5,9,10}

METHODS

This research was a comparative analytic study with cross-sectional design. A statistical analysis was conducted using nonparametric test, Wilcoxon test.¹¹ This research used biological materials stored, (BBT) mRNA Ph (+), derived from 79 patients who had been examined for BCR-ABL gene using codification of gene G6PD from April 2012 to April 2014. Data research included age, sex, hemoglobin levels, the number of leukocytes and platelets, as well as the results of comparative ratio of BCR-ABL genes with RQ-PCR method using G6PD and ABL genes.

The inclusion criteria used in sampling of this research, moreover, were biological materials stored in leukocyte mRNA of patients with Ph (+) CML less than two years with good sample stability. Their molecular comparative figures of BCR-ABL gene were examined using RQ-PCR method with the codification of G6PD gene from April 2012 to April 2014 at the Biomolecular Laboratory of Clinical Pathology Department in Dr. Hasan Sadikin Hospital. On the other hand, the exclusion criteria used in sampling of this research were all uncompleted biological materials stored in leukocyte mRNA of patients with Ph (+) CML and basic data, such as age and sex, as well as laboratory examination data about hemoglobin levels, leukocytes count and platelet count.

RESULTS AND DISCUSSION

Analysis of this research involved the characteristics of the research subject, including gender, age, hemoglobin, leukocytes, and platelets. Comparative figures of *BCR-ABL* genes used were derived from the codification of *G6PD* and *ABL* genes. In the group of BBT mRNA, the characteristics of the research subject, including gender, age, hemoglobin, leukocytes, and platelets were derived from Philadelphia positive CML patients. *BCR-ABL/G6PD* and *BCR-ABL/ABL* genes in patients then were examined as seen in Table 1.

Table 1 shows that the number of the research targets were seventy-nine samples of BBT mRNAs derived from Philadelphia positive CML patients.

Table 1. Characteristics of the research subjects

Features of Ph (+) CML	n=79
Sex	
Male n (%)	38 (48.1)
Female n (%)	41 (51.9)
Age (years)	38
Median	14–72
Range	
Hemoglobin (gr/dL)	
Median	9.7
Range	3.7–15.9
Leukocyte count (/mm ³)	
Median	47,800
Range	1,840–454,100
Platelet count (/mm ³)	
Median	378,000
Range	45,000–1,622,000

Based on the characteristics of the research subjects, it is known that the number of patients aged over 60 years was nine (9) members, consisted of six male patients and three female patients. The youngest male CML patient was at the age of 14 years old, while the youngest female one was at the age of 16 years old.

The lowest hemoglobin level was 3.7 g/dL was in a male patient aged 43 years old. Meanwhile, the highest hemoglobin level of 15.9 g/dL was in a male patient aged 44 years old.

The lowest leukocyte count was 1,840/mm³ found in a male patient aged 63 years old. The highest leukocyte count of 454 100/mm³ was a male patient aged 33 years old.

The lowest platelet count was 45,000/mm³ found in a male patient aged 34 years old. Meanwhile, the highest platelet count of 1.622 million/mm³ was a female patient aged 65 years old. And, the results of the comparative figure of *BCR-ABL* genes using the codification of *G6PD* and *ABL* genes in BBT mRNA can be seen in Table 2.

The results, moreover, show that the comparative figure of the codification of gene *ABL* was higher than the comparative figure of *G6PD* based on the median of the density of *G6PD* and *ABL*, the density of the *BCR-ABL* genes in *G6PD* and *ABL* genes and the comparative figure of *BCR-ABL/G6PD* genes and *BCR-ABL/ABL* genes.

The codification of *G6PD* gene uses a reagent LC t (9.22) of *Quantification kit (Roche)* with a standard curve corresponding to those indicated in the *insert kit*, and has an ability to detect gene types b2a2, b3a2 and e1a2.¹³ On the other hand, the codification of *ABL* gene uses a calibration curve between 102–107 mL in 5 cDNA copies. Reagents used are *Accuzol™ Total*

Table 2. The results of the comparative figure of BCR-ABL genes using the codification of G6PD and ABL genes

Variables	Examination		Z _w	P value
	G6PD	ABL		
Density				
Median	115	5,950,000	7.722	<0.001
Range	0.8–25900	397–1400,000,000		
Density of mRNA in BCR-ABL genes				
Median	2.11	1,230,000	7.574	<0.001
Range	0.0–1.710	1,060–11,900,000		
% Comparative Figure				
Median	1.93	15.37	6.477	<0.001
Range	0.0–59.69	0.04–35.677		

Note: Z_w = Wilcoxon Test

RNA Extraction Reagent Bioneer, which is capable of detecting the types of genes b2a2 and b3a2.¹⁴

The results of Mann Whitney test at the 95% confidence interval, furthermore, indicates that there were significant differences in the comparative figures between *BCR-ABL/G6PD* genes and *BCR-ABL/ABL* genes in *Philadelphia* positive CML patients with a P value of <0.001 ($P=0.005$).

Comparative figures between *BCR-ABL/G6PD* genes and *BCR-ABL/ABL* genes were obtained from the examination of the comparative figures measured quantitatively and objectively to help diagnose and monitoring of the treatment for *Philadelphia* positive CML patients. The purpose of this research then was to compare the comparative figures of *BCR-ABL* genes between using the codification of *G6PD* gene and the codification of *ABL* gene in *Philadelphia* positive CML patients. Researches on the comparative figures of *BCR-ABL* genes between using the codification of *G6PD* gene and the codification of *ABL* gene in *Philadelphia* positive CML patients actually have never been conducted in Indonesia. The population used in this research was very limited. However, this research is still expected to be preresearch on the comparative figures of *BCR-ABL/G6PD* gene and *BCR-ABL/ABL* gene. As a result, this research can be used to establish the diagnosis and monitoring of treatment for *Philadelphia* positive CML patients.

Table 1 shows that the number of female patients was higher than the male ones. According to some literatures, the prevalence of CML is found more in men than in women, but it is not influenced by geography, ethnicity and family factors.^{1,12} According to the medical records of Dr. Hasan Sadikin Hospital in 2013, a total of 39 CML patients treated consisted of 25 male patients and 14 female patients. Similarly, data from Clinical Pathology and Molecular Laboratory from April 2012 to April 2014, the number of the CML patients examined for their *BCR-ABL* genes was 174 people, consisted of 88 male patients and 86 female patients.

Thus, the characteristics of the research subjects in this research differ from the previous literatures, medical records of Dr. Hasan Sadikin Hospital, and data from Clinical Pathology and Molecular Laboratory stating CML since the number of female patients was higher than the male ones. This difference is due to the characteristics of the samples during the research period between April 2012 and April 2014 were mostly female patients with Ph (+). Similarly, a research conducted by Pane et.al.¹⁵ found

24 *Philadelphia* positive CML patients, consisted of 17 female patients and seven (7) male patients. Selection of the samples in this research was based on the molecular results of *BCR-ABL* genes with *Philadelphia* positive chromosome.

In addition, the number of people with CML disease, according to a previous literature, was 20% of all cases of adult leukemia.¹⁰ The median of age of CML patients diagnosed was between 30 and 50 years old with 12% to 30% aged 60 years or more. Although rare, CML can occur in children.^{1,12} Features of age in this research show that the age range of the patients was between 14-72 years old with a median age of 38 years. The youngest CML patient in this research was 14 years old. Similarly, a previous literature and a research conducted by Pane et.al.¹⁵ also had the age range of between 13-78 years old with the age of 13 years old as the youngest one.

In this research, moreover, hemoglobin level obtained was of 9.5 (3.7 to 15.9) g/dL. Leukocyte count found was 47,800/mm³ (18,40–454,060/mm³), while platelet count was 378,000/mm³ (45,000–16,22,000/mm³). The results of the routine blood examination in this research also generally described the clinical presentation of patients with chronic stage of CML. According to Frazer¹⁶ hemoglobin levels in patients with chronic stage of CML may be normal or decreased. Leukocyte count is usually $>50,000/\text{mm}^3$, while the platelet count may be normal, increased, or decreased.

The range of leukocyte count in this research, furthermore, was 1,840-454,060/mm³. The leukocyte count in CML patients typically was $>50,000/\text{mm}^3$. This research found a man aged 63 years with the lowest leukocyte count of 1,840/mm³, the hemoglobin level of 8.6 g/dL and the platelet count of 495,000/mm³. The low level of leukocytes is usually caused by hydroxyurea drugs ingested/taken by patients. In Indonesia, hydroxyurea can be considered as the main drug for patients with chronic stage of CML. A research conducted by Kantarjian et al¹⁷ also shows that patients in the late stage of CML previously taken *interferon alpha* drugs or hydroxyurea drugs had a median of leukocyte count of 15,000/mm³ (2,000–260,000/mm³).

The results of this research show the number of leukocytes 6.710/mm³, hemoglobin level of 3.7 g/dL and a platelet count of 87,000/mm³ obtained in a man aged 43 years. The low leukocyte count with very low hemoglobin level and declined platelet count indicated that the patient was in the crisis stage of

CML. Similarly, according to a previous literature, low leukocyte count in patients with CML indicate that they are in the blast crisis stage. A research conducted by Pane *et al.*¹⁵ shows that normal leukocyte count ranges between 9,000–205,000/mm³. Therefore, patients with leukocyte count of 9,000/mm³ can be declared in the early stages of blast crisis for 2.5 years.

In addition, in this study there were five (5) samples containing a platelet count of <100,000/mm³ (thrombocytopenia). According to a previous literature, a decline in platelet counts in leukemia is usually the result of bone marrow infiltration or chemotherapy.¹ The researcher examined the medical records, and found a sample with a platelet count of 82,000/mm³, namely a male patient aged 44 years, who were diagnosed CML in the blast crisis stage. Another sample who had a platelet count of 76,000/mm³ was a 26-year-old female patient diagnosed with CML in chronic stage + Herpes Zoster Optalmika + acute spontaneous subdural hemorrhage a/r ec temporoparietal CML. Unfortunately, the researcher did not obtain medical records of three other samples. Dr. Hasan Sadikin Hospital as a referral hospital in West Java. Similar results were obtained in a research conducted by Pane *et al.*¹⁵ showing a platelet count of 75,000/mm³ in a male patient aged 49 years old who suffered from CML in the blast crisis stage for the three (3) months, and a platelet count of 66,000/mm³ in a female patient aged 32 years old who suffered from CML in the accelerated stage.

Table 2, furthermore, shows the results of comparing the two codification of genes. The table show differences in density between using the codification of G6PD gene and the codification of ABL gene, the density of mRNA in genes BCR-ABL, and the comparative figures of BCR-ABL/G6PD and BCR-ABL/ABL. The results of Wilcoxon test in Table 2 also show differences in the range values and median values between both codifications because of the different codifications of genes used and different methods/examination procedures. The measurement of the codificaion of G6PD gene is based on fluorescent (fg of RNA in G6PD gene) as suggested in the insert kit,¹³ whereas the measurement of the codificaton of ABL gene is based on cDNA copies (5 μ L).¹⁴ The same results are also obtained in a research conducted by Beranek *et al* in 2009¹⁸ finding the density of the codification of G6PD and ABL genes as well as the density of BCR-ABL genes. Besides that, there was also a difference in the comparative figures of BCR-ABL

genes between using the codification of G6PD gene and the codification of ABL gene because of differences in methods and analysis used, different expression of the gene codification and sensitivity of the methods used.

Similar results were obtained in a research conducted by Muller *et al* in 2007.¹⁹ He compared the comparative figures of BCR-ABL/G6PD genes and BCR-ABL/ABL genes by using three (3) *LightCycler*TM and *TaqMan*TM tools. The research also used a sample of peripheral blood EDTA, namely: 2.5 mL, 5 mL and 10 mL derived from 186 healthy people. Those blood samples were mixed with serial dilutions in the ratio of 1:20 to 1:10⁶ for types of genes b2a2, b3a2, and e1a2 of positive BCR-ABL. The results show that there were six (6) tools used to obtain comparative figures in BCR-ABL/G6PD genes and BCR-ABL/ABL genes. When using the codification of ABL gene, the range value and median values obtained are different from when using the codification of G6PD gene since differences in the targeted mRNA amplification method and the codification of genes used.

Similar results were also found in a research conducted by Branford *et al*²⁰ in 2006 using three (3) different codifications of genes.²⁰ In the research, there were three (3) samples that were not detected, namely mRNA in BCR-ABL genes examined using the codifiation of ABL gene due to the ability of he codificatin of ABL gene which is only capable of detecting major translocation in P210 (a2b2 and a2b3). On the other hand, the codification of G6PD gene has an ability to detect major translocation in P210 (a2b2 and a2b3) and minor translocation in p190 (e1a2), specifically for patients with Ph (+)AL. The gene type of those three samples was e1a2 (type for Ph / + ALL) since capabilities of the codification of G6PD gene to capture gene types a2b2, a2b3 and e-1A2. Nevertheless, researchers are advised to check further to see which type those gene samples belong to. Similar results were found in a research conducted by Beranek *et al*,¹⁸ finding 80 adult subjects consisted of 65 patients with CML (26 gene types of b2a2 and 39 gene types of b3a2), eight (8) patients with Ph (+) ALL (gene type of e1a2) and seven (7) patients with suspected myeloproliferative syndromes without chromosome Ph (+). In the research, all of those eight samples suffering from Ph (+) ALL with the gene type of e1a2 gave positive results only in the codification of gene G6PD [(LC9; 22) Roche].

CONCLUSION AND SUGGESTION

Based on the results of this research, it can be concluded that there was a difference in the comparative figures of BCR-ABL genes between using the codification of G6PD gene and the codification of ABL gene in *Philadelphia* - positive CML patients without any different interpretation to the types of genes b2a2 and b3a2.

Thus, further researches are expected to use more stable *ABL* genes in determining the comparative figures of BCR-ABL genes, while G6PD gene has a great capability to detect its type. Finally, to determine the comparative figures of BCR-ABL genes, the diagnosis and monitoring of treatment of patients with Ph (+) CML should be done with the codification of the same gene.

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