Detection of bcr-abl Fusion Gene Expression in Chronic Myelogenous Leukemia At Sardjito Hospital: Case Reports

Elizabeth Henny Herningtyas1, Sofia Mubarakia Haryana2, Indwiani Astuti3

1Clinical Pathology Department, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia
2Histology Department, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia
3Pharmacology Department, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia

Abstract

Leukemia is hemology malignancy caused by excess proliferation of hematopoietic or lymphoid cells. Leukemia cases in Indonesia were about 3.7 per 100,000 with mortality rate 83.6% and diagnosed based on FAB classification. The fact, WHO classification 2000, used worldwide based on cytogenetics and molecular biology profile, can define the clonal diseases more precisely and to choose the adequate therapy. CML case is about 15% of all adult leukemia cases and most of the cases are related with 1(9;22)(q34;q11). The aim of this report is to show the rare case of CML that has been examined for bcr-abl fusion gene. Blood sample was obtained from CML patients diagnosed by hospital doctors based on FAB classification. Mononuclear cell was separated by ficoll-hypaque gradient centrifugation, then RNA was isolated by Trizol and converted to cDNA by RT reaction. Beta-actin gene was used as internal control and bcr-abl gene was amplified by nested PCR. We reported CML cases classified as: t(9;22)(q34;q11) and t(9;22)(q34;q11) type based on FAB classification with post therapy for the second CML At molecular level, bcr-abl fusion gene found at the second case with longer product than positive control.

Keywords: leukemia, CML, fusion gene, bcr-abl

Introduction

Leukemia is a blood disease resulting from the neoplastic proliferation of hematopoietic or lymphoid cells that can affect everyone, every age and sex (Bain, 2003). Leukemia prevalence varies at many countries, with range between 1-4.8 per 100,000 populations. Prevalence of leukemia cases in Indonesia was around 3.7 per 100,000 with mortality rate 83.6% (WHO, 2001). Leukemia diagnosis in Indonesia is based on French-American-British (FAB) classification, even though World Health Organization (WHO) classification (2000) emphasized that cytogenetic and molecular biology detection are very useful to determine the disease clonality and to choose precise therapy. Chronic Myelogenous Leukemia (CML) case is about 15% of all adult leukemia cases and account for 20% of leukemia death (Jandl, 1996). In FAB classification, CML is included in myeloproliferative disorder, and clinically, it has 3 stages of disease: chronic, accelerated, and blast crisis phase. These three stages have different peripheral blood and bone marrow characteristic and different morphology pattern. The prognosis is getting worse for the extended stage (Bain, 2003; Jandl, 1996).

CML has known as the first type of leukemia that shown cytogenetic abnormality (9;22)(q34;q11) translocation, namely Philadelphia chromosome, and expressed bcr-abl fusion gene at molecular level (Bain, 2003; Jaffe, 2001). The expression of this fusion gene could be detected at mRNA level by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) technique (Weafer, 1999). In WHO classification (2000), CML is also grouping as myeloproliferative disorder but with different subgroup. There are 2 subclasses based on cytogenetic and molecular characteristic: first, with Philadelphia chromosome and expressed bcr-abl fusion gene and second, without Philadelphia chromosome but also expressed bcr-abl fusion gene (Bain, 2003).

The fusion gene could influence disease progress in leukemia patient. CML patient with positive bcr-abl fusion gene have relapse possibility during 1-3 months after chemotherapy comparing with negative bcr-abl that could survive for several years after chemotherapy (Lowenberg, 1999; Dukker, 2001; Serrano et al., 2000).

Diagnosis and classification of leukemia by molecular technique based on genetic abnormality and clonal changing are never reported in Indonesia, especially at Sardjito Hospital Yogyakarta. This study was held to applied molecular technique in clinical setting by detect the expression of bcr-abl fusion gene at CML cases. We reported 2 CML cases with different clinical type and treatment, one was without chemotherapy case and the other was post therapy case that have been checked for the bcr-abl fusion gene by RT-PCR method.

Material and method

All reagents and primers in this study were provided by Hemaatology/Oncology Subdivision, International Centre for Medical Research (ICMR), Kobe University, Kobe, Jepang. The research was held at Clinical Pathology Instalation Sardjito Hospital, Tumor Biology Laboratory Histology Department Faculty of Medicine GMU Yogyakarta, Biochemistry Laboratory Inter University Centre GMU Yogyakarta and ICMR, Kobe School of Medicine, Kobe University, Kobe, Jepang from July 2003-Mei 2004.

The sample was mononuclear cell isolated from inward patients Sardjito Hospital that diagnosed as CML based on FAB classification and gave the informed consent. Mononuclear cell was isolated by ficoll-Hypaque gradient centrifugation from 5-10 mL heparized peripheral blood then kept at -80°C as frozen pellet until the RNA isolation. Total RNA was isolated from the mononuclear cell using Trizol reagent (Life Technologies), according to the manufacture recommendation. Briefly, approximately 107 cell of frozen pellet were thawing and mix with 1 mL of Trizol then let for 10 min at room temperature. After centrifugation at 12,000 rpm for 10 min in a minicentrifuge, the supernatant was treated with 0.2 mL chloroform then precipitated with 0.5 mL isopropanol. The pellet was washed with 70% cold ethanol and dissolved in 100 µL sterile water. The purity and concentration of the RNA preparation were spectrophotometrically measured. The cDNA was made from RNA by a reverse transcriptase reaction with random primers and beta-actin gene was used as internal control. Primer for beta-actin gene were 5′-ACT GAG TGA GCT GTC GTA CGT CGC CC-3′ and 5′-AGC GAA GAA GCT GTG CTA CTC TGC CGC CC-3′ (with sequence 5′-3′ TAC AGT GTG CCG CCA GAC AG). The tube containing amplification mixture subjected to 30 cycles on a thermal cycler (Takara thermocycler, Japan) with the following programs: 1×5 min precycle at 94°C for 5 min, for the next cycle denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and the last extension at 72°C for 9 min.

bcr-abl fusion gene was amplified by nested PCR with following primers: ABL-1 (with sequence 5′-3′: GCC CCA TGG TAC CAG GAG TG), ABL-2 (with sequence 5′-3′: GTT TCT CCA GAG TGT TGA CTG), BCR-1 primer (with sequence 5′-3′: GCC TCT CCC TGA CAT CCG TG), and BCR-2 (with sequence 5′-3′: GAG GCT GCA GAT...
GCT GAC CAA C). The amplification programs were: 24°C 1 min precycle at 94°C, 35°C 1 min at 94°C, 1 min at 72°C followed by a final extension incubation at 72°C for 10 min. The target fragment was 371 bp.

The presence of PCR product was determined by electrophoresis of 7 mL of the reaction product in 2 percent Agarose gel, with tris acetate-electrophoresis buffer TAE (0.04 mol Tris, 0.001 mol EDTA, pH 7.8) and a 100 bp DNA ladder (Gibco) as molecular marker. The electrophoresis was visualized by UV light and documented by Polaroid film.

Result and discussion
Case 1
a. Patient characteristic and history
Leukemia cell was isolated from a 69 years old woman that admitted to hospital cause of weakness and dyspnea. The weakness has been suffered since 2 months ago with fullness and distention of the abdomen, then many lumps appeared at her neck, axilla and inguinal. When admitted to hospital, physical examination revealed anemia, hepatosplenomegaly and multiple lymphadenopathy at the neck, axilla and inguinal and also sternal tenderness.

b. Laboratory examination
At admission, blood test showed leukocytosis (WBC 196 x10^9/L), anemia (Hb 51 g/L), and thrombocytopenia (PLT 50 x10^9/L) with complete leukocyte spectrum at differential count (from blast cell to neutrophil), with promyelocyte predominance (33.6%) at peripheral blood and bone marrow smear. Peripheral blood morphology showed elevated blast count (9%) and hypercellularity at bone marrow examination with higher blast count (26%) (Figure 1). There is no dysplastic morphology at all cell lineages in bone marrow (erythocyte, leucocyte or megalokaryotic lineage). Bone marrow cytochemistry staining pointed to myeloid type with diffuse positive result for Periodic Acid Schiff (PAS) staining at granulocyte series above myeloblast series. Positive result was also found in Sudan B Black (SBB) staining (Figure 2).

![Figure 1. Bone marrow cellularity and leukocyte cell morphology in case 1 (May-Grünwald Giemsa staining, 10x and 100x objective magnification). Note: a=myeloblast, b=myelocyte, c=metamyelocyte, d=promyelocyte](image1)

![Figure 2. Bone marrow cytochemistry staining of case 1 (PAS and SBB staining, 100x objective magnification). PAS= Periodic Acid Schiff, SBB= Sudan B Black. Note: a=myelocyte, PAS (+) diffuse; b=myeloblast, PAS (+) diffuse; c=metamyelocyte, SBB(+); d=myeloblast, SBB(+)](image2)

During hospitalization, the patient got cephalosporin therapy and hydroxyurea but after 2 days, she developed gastric stress ulcer's sign as melena and also showed renal failure with increasing BUN and serum creatinine (BUN 32.8 mmol/L, creatinine 2.01 mmol/L). Gastric ulcer can be overcome by anti-ulcer therapy but the patient developed hyperuricemia (urate acid 14.4 mmol/L). Anemia was overcome by 8 kolf of Packed Red Cell (PRC) transfusion. No remission induction treatment was given for leukemia therapy. After 9 days treatment, the families asked to take care her at home but 2 days later she died at home.

c. Molecular examination
There was no bcr-abl fusion gene expression that found by RT-PCR with primers for b3 exon of bcr gene and a2 exon of abl gene (b3a2). In fact, bcr-abl fusion gene has several different breakpoint such as M-BCR, m-bcr and m-bcr that gave different fusion proteins (Vardiman et al, 2001, Bain, 2003). As a result, this different breakpoint or exon/intron different breakpoint can not be revealed by primers used in this study; 2) there is no bcr-abl fusion gene in this case. Several researcher explained that bcr and abl gene rearrangement resulted bcr-abl fusion gene was found in 90-95% of CML cases in the world, so only 5-10% of CML case was negative for bcr-abl fusion gene (Bain, 2003, Vardiman, 2001). There are 3 possibilities of myeloproliferative disorder without bcr-abl fusion gene: atypical CML, CML (Chronic Neutrophilic Leukemia) and CMML (Chronic Myelomonocytic Leukemia) (Kuzock, et al, 2001).

Based on diagnosis criteria of those 3 possibilities, CML and CMML possibilities can be excluded because in CML diagnosis criteria, the case should have stab and mature neutrophil more than 80%, immature granulocyte less than 10%, myeloblast less than 1% of the peripheral leukocyte differential (Imbert, et al, 2001). Diagnosis criteria for CMML are persistent monocytosis more than 1x10^9/L, blast cell less than 20% of peripheral and bone marrow leukocyte count (Vardiman, et al, 2001)

This case, based on WHO classification, was diagnosed as atypical CML because of leucocytosis in peripheral blood with increasing mature and immature neutrophil percentage, dysgranulopoiesis, the absence of bcr-abl fusion gene, neutrophil precursor more than 10%, no basophilia or monocytosis. The accelerated phase supported by the infiltration signs in lymph node that frequently found in accelerated phase or blast crisis CML (Kjeldsberg, et al, 1995), and increasing blast cell percentage but less than 30% in bone marrow and less than 10% in peripheral blood (Bain, 2003, Vardiman, 2001). Atypical CML have worse prognosis than typical type (Kjeldsberg, et al, 1995; Bain, 2003) especially with accelerated phase as shown in this case.
This case is a very rare case, especially in Indonesia and Yogyakarta because until now there is no report or publication about atypical CML accelerated phase with negative bcr-abl fusion gene expression.

**Case 2**

a. **Patient characteristic and history**

A 41 years old man complained about bleeding in his mouth mucosa, under his arm skin and a lump on his thigh. He checked himself to Sardijto Hospital and admitted for 3 days. There were no organomegaly or lymphadenopathy signs at admission but blood tests revealed anemia (Hb 8.9 g%), leukocytosis (AL 140×10⁹/L), and trombocytopenia (9×10⁹/L). Peripheral and bone marrow cell morphology showed CML chronic phase pattern based on leukocytosis with complete cell spectrum dominated by promyelocyte (20%), basophilia pattern, eosinophilia and bone marrow hypercellularity (erythroid: myeloid ratio = 1:2.5). Elevated erythroid series and nucleated erythrocyte cell (4%) was also found with normal megakaryocyte morphology. Philadelphia chromosome was found at cytogenetic test.

Patient was treated with Hydrea for 1.5 months then the dose was tapering off and he was scheduled for bone marrow transplantation in Singapore. But, the lack of donor causes the therapy changing to imatinib mesylate for 7 months. Patient can reach complete hematological remission (CHR) until present. Imatinib mesylate therapy was still continued although there was a slight side effect as asthralgia. The patient's white blood count increased in every reducing dose. The peripheral blood examination was performed routinely every week at Sardijto Hospital to monitor the patient condition. The results and morphology was in normal range (Figure 4) with WBC range between 9-11×10⁹/L. When involved in this study, patient was still in therapy and in good condition.

**Molecular examination**

Fusion gene examination revealed positive result for bcr-abl fusion gene with the amplification product size about 400-500 bp, longer than the positive control that has length 371bp (Figure 3).

In the second case, classical CML diagnosis was supported by leukocytosis with increasing percentage all myeloid cell spectra in peripheral and bone marrow, the existence of basophilia, eosinophilia and Philadelphia chromosome. Hydroyxurea is a chemical agent that can inhibit DNA synthesis in cell by inhibiting ribonucleotide reductase enzyme then disturbing deoxyribonucleotide formation and dNTP pool in cell (Adams and Lindsay, 1967; Bianci, et al., 1986). Hydroyxurea therapy is a recommended therapy for CML and other myeloproliferative disorder (Cortelazo, et al., 1995; Silver, et al., 1999) and known to give better short term survival than other therapy such as busulfan (Silver, et al., 1999), bone marrow transplantation (Gale, et al., 1998), or combination therapy with interferon. (The Benelux CML Study Group, 1996). But, for long term survival, bone marrow transplantation is more effective especially in youth group (Gale, et al., 1998; Silver, et al., 1999).

In this patient, hydroyxurea is chosen as early therapy to reduce the leukemia cell proliferation rate, as preparation for bone marrow transplantation and gave good response as shown by complete hematological remission (CHR). But, the lack of bone marrow transplantation donor, the therapy is switched to imatinib mesylate.

Imatinib mesylate is a 2-phenylaminopirimidine derivate, with brand name Gleevec (STI571), has specific inhibition effect to fusion protein that encoded by bcr-abl gene (Savage and Antman, 2002; Holtz, et al., 2002). Imatinib mesylate has been proven as leukemia cell proliferation inhibitor for progenitor cells (Holtz, et al., 2002), for newly diagnosed CML patient (Kantarjian, et al., 2003), for chronic phase, accelerated or blast crisis during 2nd phase clinical trial (Braziel, et al., 2002; Talpaz, et al., 2002) and for CML with secondary chromosomal abnormality (Mohamed, et al., 2003) but with different percentage successfulness of hematological remission, cytogenetic and survival or relapse rate also intolerance side effect (Druker, et al., 2001).

The problem found in this patient is persistence of bcr-abl fusion gene after hydroyxurea and bcr-abl inhibitor therapy at RT-PCR checking, although hematologically the patient reached complete hematological remission. This bcr-abl persistence indicated that leukemia clone in this patient can not be eradicated by bcr-abl inhibitor and resulted resistance to imatinib mesylate.

Several investigitors have reported imatinib mesylate resistance and the mechanism are predicted as multifactorial. The following resistance mechanism has been reported: 1) bcr-abl gene amplification, 2) point mutation in ATP-binding pocket in ABL kinase domain like mutation at position 315 that change tyrosine to leucine, at position 1127 that change glycine to lysine (Hofmann, et al., 2002) and several other mutation (Barthe, 2001; Branford, et al., 2002) that change the conformation of bcr-abl and prevent imatinib binding to bcr-abl protein, 3) increasing of AGP (alpha-glycoprotein) plasma concentration that binded with imatinib and blocked imatinib capability to inhibit kinase activity of BCR/ABL (Marcuci, 2003).

Several new chemical agents include in piridopirimidin (PD180970 and PD166526) are investigating and developing to replace imatinib therapy in resistance cases (Rosee, et al., 2002; Huron, et al., 2003).

In the case, longer bcr-abl product (around 400-500bp; positive control 371 bp) has been detected (Figure 3). It means there were several base additions to patient bcr-abl gene. The addition might be caused by gene amplification with very short distance or only the part of gene meanwhile point mutation does not cause length changing but might change coded protein conformation especially if the point mutation change the ABL-binding pocket sequence. To determine the exact changing in this bcr-abl product, a further investigation and sequencing is needed.

The patient prognosis can not be determined but routine monitoring and Gleevec therapy are continuing.

**Conclusion**

We reported CML cases classified as atypical (case 1) and typical (case 2) type based on FAB classification with post therapy for the second CML. At molecular level, bcr-abl fusion gene found at the second case with longer product than positive control.

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**References**


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The effect of horse milk lactoferrin on mice cellular immune response: Lymphocyte proliferation and interleukin production

Nurliyani1, Mohamad Adnan 3, Wayan Tunas Artama2, and Zuheid Noor2

1 Faculty of Animal Science, Gadjah Mada University
2 Faculty of Agriculture Technology, Gadjah Mada University
3 Faculty of Veterinary Medicine, Gadjah Mada University

Abstract

Lactoferrin (Lf) is an iron-binding glycoprotein, has been known as a nonspecific immunomodulator. The aims of this research were to study the effect of horse milk lactoferrin on mice lymphocyte proliferation and interleukin (IL-2, IL-4) production. Female Balb/c mice 6-8 weeks old, fed horse milk lactoferrin 1.0 mg/mice/day, in assay period of 28 days. On the 7th, 14th, 21st and 28th day, lymphocyte proliferation was assayed with MTT. IL-2 and IL-4 were analyzed by ELISA. Result indicate, lymphocyte proliferation of mice fed horse milk lactoferrin was higher than the control and bovine milk lactoferrin on the day of 14th, whereas lymphocyte proliferation of the 28th day of mice fed bovine milk lactoferrin was lower than horse milk lactoferrin and the control. Level of IL-2 and IL-4 in lymphocyte culture, and IL-4 in serum of mice fed horse milk lactoferrin, were higher than the control, whereas serum IL-2 was undetectable.

Keywords: Horse milk lactoferrin, lymphocyte proliferation, interleukin production

Introduction

Horse milk and horse milk products have been known as health food. However, the scientific basis to support this practice has not been proofed, particularly the horse milk lactoferrin as an immunomodulator.

Lactoferrin (Lf) is an iron-binding 80 kDa glycoprotein, belongs to the transferrin family that is present in leukocytes, and many exocrine secretions (e.g. milk, saliva, tears, mucosal and genital secretions) (Karthikeyan et al., 1999). Lactoferrin is an example of a minor milk protein (Stejins, 2001), has a very broad range of biological functions relating to the host defense system and thus, is a multifunctional protein (Shimazaki et al., 1998). Biological activity of lactoferrin is directly related to its three major structural properties: 1) it is organized in two homologous lobes, with one iron-binding site each, 2) the presence of glycosylation sites, and 3) the highly basic N-terminus mediates the binding to several eukaryotic and prokaryotic structure (Aguila and Brock, 2001). The biological function of lactoferrin e.g. as antimicrobial, antiviral, antioxidant, and an immunomodulator (Brink, 2000).

Domain arrangements in the transferrin proteins to be an important structural feature related to their specific biological functions. Based on the structures of transferrin, it can be stated that the native apoprotein of transferrin family adopt three forms: 1) with both the N and the C lobes in closed forms, as observed in horse apolactoferrin, 2) with the N lobe open and the C lobe closed, as observed in human apolactoferrin, and 3) with both the N and the C lobes open, as found in duck apotransferrin (Sharma et al., 1999). Apolactoferrin and partially saturated N-lobe forms being predominant in vivo (Aguila and Brock, 2001).

The content and composition of lactoferrin glycans greatly varies according to species (Karthikeyan et al., 1999, McAbee and Walsh, 2000), and the glycosylation sites is related to resistance to mucosal protease (Aguila and Brock, 2001). The amino acid