

HES-1 and CEBPA mRNA in Chronic and Late Phase (accelerated and blast crisis) of Chronic Myeloid Leukemia (CML) Patients

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Abstract

Chronic Myeloid Leukemia (CML) is a myeloproliferative disorder of hematopoietic, characterized by Philadelphia chromosome containing BCR-ABL fusion gene. The gene encodes protein with constitutive tyrosine kinase activity result in myeloid proliferation and leads to form an early phase of CML called chronic phase. Unsuccessful treatment will lead to progression of the disease into late phase (accelerated and blast crisis). The mechanisms involving disease progression are still poorly understood. It is assumed that additional genetic event involves in differentiation blocking of myeloid progenitor cells, such as Hes-1 overexpression and CEBPA down regulation. However, study on the expression of these genes in CML patient's samples is still limited. This study aims to measure Hes-1 and CEBPA mRNA in chronic and late phase of CML patients. The peripheral blood mRNA level of Hes-1 was measured in CML patient's sample with BCR-ABL positive both in chronic phase (n=61) and late phase (n=17) using qRT-PCR with GAPDH as internal control. Hes-1 mRNA was statistically higher (p value=0.0) in the chronic phase (mean \pm SD=97.8 \pm 236.6) compared to those in late phase (mean \pm SD=8.5 \pm 30.7). In addition, even though CEBPA expression in chronic and late phase were not statistically different (p value=0.1), those in chronic phase (mean \pm SD=5.2 \pm 16.0) were generally higher compared to those in late phase (mean \pm SD=1.7 \pm 2.4). Hes-1 expression upregulated in 70.5% of chronic phase patients and in 17.6% of late phase patients, whereas CEBPA expression down regulated in 42.6% of chronic phase patients and in 47.1% in late phase patients. High standard deviation, particularly in mRNA Hes-1 gene expression measurement of the chronic phase, indicated the presence of individual variations in the sample that might be influenced by other genetic factors. This study found that Hes-1 mRNA is significantly higher in peripheral blood of chronic phase than blast crisis CML, whereas CEBPA mRNA is not different.

Keywords: Hes-1; CEBPA; Chronic phase; Late phase; Chronic myeloid leukemia

Introduction

Chronic Myeloid Leukemia (CML) is a hematological malignant disease with a high rate incidence. CML is closely related to BCR-ABL fusion gene [1,2], indicated by approximately 90-95% of all cases have BCR-ABL fusion gene. BCR-ABL fusion gene is occurred due to a reciprocal translocation between chromosomes 9 and 22, which will form the Philadelphia chromosome. The translocation causes the fusion of BCR-ABL gene that encodes oncogenic protein P210BCR-ABL [1,3,4]. BCR-ABL has a constitutive tyrosine kinase activity, which will lead to the activation of intracellular signaling pathways for myeloid cells proliferation and survival [1,5,6]. CML is divided into three phases, which are chronic phase, accelerated and blast crisis. Accelerated and blast crisis phase are also called as late phase. World Health Organization (WHO) characterizes the phases based on the number of immature myeloid cells (blast cells) that accumulated in the blood or bone marrow. Chronic phase patients usually have less than 10% blast cell counts; accelerated phase patients have 10-20% blast cell counts; and blast crisis phase patients have more than 20% blast cell

counts. Blast crisis is the terminal phase of CML and occurs due to the blocking of myeloid cells differentiation (differentiation arrest) [7].

The molecular pathways related to the disease progression from chronic phase into late phases (accelerated and blast crisis) are still poorly understood. Secondary genetic abnormalities or mutation other than BCR-ABL are assumed to play roles in the CML progression [7,8]. In addition, the resistance of tyrosine kinase inhibitor therapy also can accelerate the disease progression [5].

Particularly, myeloid differentiation is induced by CEBPA gene which encodes the C/EBP- α (CCAAT-enhancer binding protein alpha) [9], a protein that plays a role to induce genes transcription related with myeloid differentiation. In CML cases, CEBPA expression is inhibited by variety of genes, including BCR-ABL, Hes-1, and hnRNP-E2, which result in the blocking of myeloid differentiation and disease progression from chronic phase into late phase [9,10]. Hes-1 gene (hairy enhancer of split 1) is a transcriptional repressor of multiple genes, one of them is CEBPA. Previous studies using *in vitro* and *in vivo* in mice model approaches indicated that Hes-1 is overexpressed in blast crisis CML phenotype. Hes-1 overexpression is assumed to inhibit CEBPA expression result in the blocking of myeloid

differentiation [9-12]. However, there are still lacks of evidence of these conditions in humans. Study in CML patients showed that only 8 of 20 patients with blast crisis blood samples have an enhancement level of Hes-1 gene expression compare to healthy individuals [12]. Whereas, studies of CEBPA gene expression in CML have been only conducted using in vitro and in vivo in mouse model approaches and these have not been performed in human yet [9]. This study, therefore, aims to measure the mRNA expression of Hes-1 and CEBPA in patients with chronic phase and late phase (accelerated and blast crisis) CML.

Method

Samples

This study used peripheral blood total RNA samples of CML patients from RSUP Dr. Sardjito (2010-2015) that initially diagnosed in chronic phase (n=61) and late phase (n=17). The patients did not suffer from other malignancies and at the age range of 18-65 years old. The samples have been proved as BCR-ABL positive. This study has been received an approval from the Ethics Committee of Faculty of Medicine (No. KE / FK / 640 / EC / 2016).

Hes-1 and CEBPA mRNA measurement using qRT-PCR

Prior to the measurement of Hes-1 and CEBPA mRNA, the total RNA of each sample was underwent cDNA synthesis using transcriptor FS cDNA synthesis katalog No. 04896866001. The measurement of Hes-1 and CEBPA mRNA were performed using 7500 Fast Real Time PCR (Applied Biosystems) machine. The following primer pairs were used: 5'GCA CCG TCA AGA AGG CTG AC 3' (forward) and 5' TGG TGA CAG AGA CGC TGG A 3' (reverse) for GAPDH; 5'GGC GAA AGG GAC ACT TAG TG 3' (forward) and 5'CGA CCT TTC GCT GTA GCA TT 3' (reverse) for Hes-1; and 5'CGA TTC CCT GTA GCT GCA TT 3' (forward) and 5'CCC ATG GCC TTG ACC AAG GAG 3' (reverse) for CEBPA. Reaction was subjected to 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 15 seconds and of 60°C for 1 min; and 1 cycle of 95°C for 30 seconds.

Statistical analysis

The data obtained in this study were numerical data of R (fold change) value representing the mRNA relative mean of target genes (Hes-1 and CEBPA) toward GAPDH as internal control. These data were analyzed using SPSS.21 software to compare the mean value of R (fold change) between chronic phase [13-20] and late phase groups. Kolmogorov-Smirnov test was used to test normality of the data, while unpaired t-test was used to test significance differences between the two groups. The significance was marked with p value <0.05.

Result

The number of samples used in this study was 61 samples for chronic phase and 17 samples for late phase with the age range of 18-65 years old which divided into three age categories (Table 1). Most of the chronic phase patients were found at the age range of 18-39 years old, which constitute of 47.5% (29/61). Late phase patients were also mostly found at the age range of 18-39, which constitute of 52.9% (9/17) (Table 1). The percentage of male and female subjects in chronic phase was almost equal, which were 54.1% and 45.9%, respectively. Whereas, in the late phase, the percentage of male patients were higher than female this was 70.6% and 29.4%, respectively (Table 1).

Characteristic	Chronic phase (n=61)	Late phase (n=17)
Age		
18-39	29 (47.5%)	9 (52.9%)
40-54	22 (36.1%)	4 (23.5%)
55-65	10 (16.4%)	4 (23.4%)
Gender		
Male	33 (54.1%)	12 (70.6%)
Female	28 (45.9%)	5 (29.4%)

Table 1: Subject characteristics of patient's samples in chronic phase and late phase of CML

The raw data of Hes-1 and CEBPA mRNA level in this research were not in normal distribution. High individual variations can be seen in the Hes-1 mRNA in chronic phase (Figure 1) with a maximum value of 1264.9 and a minimum value less than 0.001 (data not shown). High individual variations were also verified with high standard deviation, which was 236.6 (Table 2). Hes-1 mRNA also had individual variations, even though it was not as high as the one in chronic phase (Figure 1A) with maximum values of 127.2 and minimum value less than 0.001 (data not shown). Standard deviation value of Hes-1 mRNA was 30.7 (Table 2). The individual variation of CEBPA mRNA in the chronic and late phase was not as high as the one in Hes-1 (Figure 1B). It was verified by lower standard deviation which was 16.0 in chronic phase and 2.4 in late phase (Table 2). CEBPA expression in chronic phase had a maximum value of 122.3 and minimum value of less than 0.001, while in late phase, the maximum value was 4.9 and minimum value was 0.2 (data not shown).

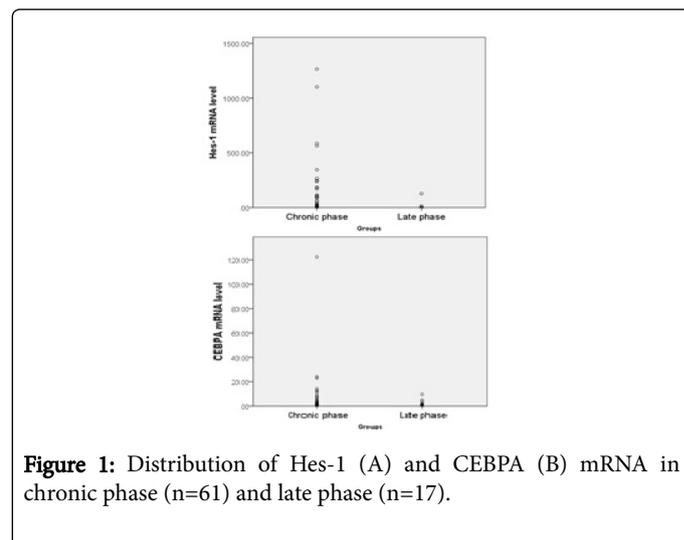


Figure 1: Distribution of Hes-1 (A) and CEBPA (B) mRNA in chronic phase (n=61) and late phase (n=17).

The mean value of Hes-1 mRNA in chronic phase (97.8) was higher than those in the late phase (8.8) (Table 2). Mann Whitney test showed that the mean value of Hes-1 mRNA in those two groups were statistically different (p=0.0) (Table 2). Unpaired t-test showed that the mean value of CEBPA mRNA in both groups were not statistically different (p=0.1), even though the expression in chronic phase (5.2) was higher than the one in late phase (1.7) (Table 2).

Target gene	Group	R=2 -ΔΔCP	p-value
Hes-1	Chronic phase	97.8 ± 236.6	0.0*
	Late phase	8.5 ± 30.7	
CEBPA	Chronic phase	5.2 ± 16.0	0.1
	Late phase	1.7 ± 2.4	

Statistical analysis was performed using unpaired t-test for CEBPA and Mann Whitney test for Hes-1* p <0.05 indicates statistically different

Table 2: The mean value of Hes-1 and CEBPA mRNA in chronic phase and late phase of CML patient's sample.

In every amplification process, Hes-1 and CEBPA mRNA of healthy individual was used as a healthy control. Compare to that in healthy control, Hes-1 mRNA expression was upregulated in 70.5% (43/61) patients in chronic phase and in 17.6% (3/17) patients in late phase. The sample percentage with downregulated CEBPA expression in chronic and late phase was almost equal, which were 42.6% (26/61) in chronic phase and 47.1% (8/17) in late phase (Table 3).

Groups	Upregulated (%)	Downregulated (%)
	Hes-1	CEBPA
Chronic phase	70.5	42.6
Late phase	17.6	47.1

Note: The upregulated or downregulated gene expression was compared with gene expression in healthy donor.

Table 3: The percentage of sample with Hes-1 up regulation and CEBPA down regulation.

Discussion

CML cases that initially diagnosed in chronic phase were most prevalent in patients with age range of 18-39 years old with percentage of 47.5%, while CML cases that initially diagnosed in late phases also were most prevalent in the patients with age range of 18-39 years old with percentage of 52.9% (Table 1). Several studies mentioned the CML cases were rare in children or young age. In Asian countries, the average age of patients who initially diagnosed with CML were in 40's years old, which is lower than those in Western countries, most prevalent cases are diagnosed in the age range of 55-80 years old [1,20]. In this study, the CML cases that initially diagnosed in the age range of 40-54 years old were 36.1% in chronic phase and 23.5% in late phases. These numbers were higher than the percentage of CML cases in the age range 55-65 years old, especially in chronic phase (10%).

These data support earlier statement that the CML cases in Asian countries were quite commonly found in the 40's, although in fact there were many cases in Indonesia that found at a younger age. In addition, the percentage of cases which found in men was higher than those in women both in chronic phase and late phase. However, the percentage of chronic phase was almost balance. Several studies also mentioned that the ratio between male and female were 1.3-2.2:1 [1,20]. The results of this study support the statement that the ratio of patients with chronic phase between male and female were 1.2:1 (54.1%:45.9%), whereas in the late phase it was 2.4:1(70.6 %: 29.4%).

However, no further studies mentioned that neither age nor gender is one of factors that, directly or not, affect the CML. It indicates that further study is needed to support the statement.

This study also showed that there were considerable variation in the Hes-1 and CEBPA mRNA level (Figure 2). Hes-1 expression was detected very high in several samples reaching value of 1264.95, whereas in other samples, Hes-1 expression was very low to detect. Besides, CEBPA expression was detected in the values of 122.35, whereas in other samples, the expression also was very low to detect (data not shown). It indicates that there were likely individual variations in the samples. In CML cases, result's variation can be caused by secondary genetic abnormalities which were different in each patient's sample [17]. In addition, the microarray analysis in previous study showed that there was heterogeneity of gene mutation in CML cases. From the results of those studies, the genes that consistently mutated in CML cases are still unknown besides BCR-ABL. Some types of tumor cells with the same phenotypic could also have different types of gene, so that it caused the therapy for late phase of CML was more difficult to do [21].

Hes-1 is a transcriptional repressor and one of its target is CEBPA. Hes-1 plays a role to maintain the cells in immature state by inhibiting transcription of CEBPA which play a role to induce cell differentiation process. One of the main causes of CML chronic phase develops into late phases (accelerated and blast crisis) is the inhibition of myeloid cell differentiation processes or called as differentiation arrest. Hes-1 and CEBPA were assumed to play as a key role on the processes. Previous studies using in vitro and in vivo in animal model showed that Hes-1 expression increased in CML blast crisis phenotype [22-25]. Study on patient's samples also showed that Hes-1 expression increased in 40% of blast crisis patient's samples compared to healthy control. In this study, it was found that only 17.6% of Hes-1 mRNA of blast crisis sample were increased compare to healthy control, whereas 70.5% of Hes-1 mRNA level were increased in chronic phase. In general, the results obtained in this study showed that Hes-1 mRNA level expression was statistically higher in chronic phase compared to those in the late phase (Table 2). None of previous study compared the Hes-1 expression in chronic phase and blast crisis. It assumed that Hes-1 probably not the only key genes that play a role in inhibiting differentiation processes in CML. However, it is still unclear whether Hes-1 has a role in the development of CML or not. So, further study is needed to answer this question.

Based on several studies, the role of Hes-1 in the development of hematological malignancies remains unclear. Several studies mentioned Hes-1 act as an oncogene while several others mentioned that Hes-1 act as a tumor suppressor gene [13-15]. Previous study demonstrated that Hes-1 act as a tumor suppressor gene in the AML (acute myeloid leukemia) cases. Hes-1 was referred to inhibit tumor cell proliferation by increasing the expression of the p21 gene which is an inhibitor of cyclin, so that it will induce cell cycle arrest [15]. In addition, Hes-1 was also act as a tumor suppressor by inhibiting HDM2 that will induce p53 expression. P53 gene is a tumor suppressor gene that plays a role to regulate cell cycle when DNA damage occurred. P53 expression was inhibited by HDM2 or MDM2 by the binding with those genes [22].

Contrarily, other studies mentioned that Hes-1 has a role in the development of tumor cells in T-ALL through NFκB pathway. Hes-1 induced NFκB activities by inhibiting expression of IκB [13]. In addition, several studies in T-ALL mentioned about the role of Hes-1 in tumorigenesis. Ectopic induction of Hes-1 was capable to cause

tumor cell development in mice models. The mechanism of Hes-1 to induce the development of T-ALL was by increasing the expression of c-myc and Nrarp which is an oncogene [23]. Hes-1 also has a role in the development of several kinds of cancer by maintaining cancer's stem cell, inducing metastasis and resistance of some antitumor drugs [10]. Hes-1 transduction followed by high expression of BCR-ABL could increase the expansion of myeloid progenitor cells in vitro and in vivo [12]. Based on these statements, Hes-1 may play a role in regulating a variety of molecular pathways, such as proliferation, differentiation, and apoptotic pathways.

CEBPA as the target gene of Hes-1 was also assumed to be the genes that have a major role in the development of CML. CEBPA is a gene that plays a role to induce the differentiation of myeloid cells [9,16,24-26]. Based on previous *in vitro* and *in vivo* studies, CEBPA expression could be inhibited by BCR-ABL through MAPK-hnRNP-E2 pathway in CML blast crisis [9]. The results obtained in this study indicated that CEBPA expression in chronic phase and late phase was similar. Even though CEBPA mRNA in chronic phase was higher than those in late phase, but it was not statistically difference.

Other than CEBPA, several genes was assumed to play a role in the myeloid differentiation blocking. Deletion of IKZF1, a gene encoding a protein called Ikaros, could cause differentiation blocking in CML. In addition, NUP98-HOXA96 and AML1-EVI1 gene mutations also had been known to have a role in myeloid differentiation blocking, but it was only proven in AML cases [17]. NPM1 (nucleophosmin 1) was also mentioned to has a role in leukemogenesis by inhibiting myeloid cells differentiation in AML [27]. In addition, c-Myc and c-Myb oncogene which was often found in many types of cancer may also play a role in the myeloid differentiation blocking [25,28].

Nevertheless, many studies mentioned that the main regulator of myeloid differentiation was CEBPA. CEBPA expression was regulated by BCR-ABL through MAPK-hnRNP-E2 pathway, so that when the expression of BCR-ABL in CML increased, it would down regulate CEBPA expression [9-30]. However, other than CEBPA, hnRNP-E2 also targeted some other genes such as RhoGTPase Cdc42 and S100A9, which also play a role in myeloid differentiation. In addition, expression of hnRNP-E2 could also be inhibited by other genes such as UPF1. Previous studies proved that UPF1 knockdown caused hnRNP-E2 overexpression. Hence, it will decrease the expression S100A9 which also an important regulator for myeloid differentiation [30].

This study did not perform an examination for the other genes expression involved in the direct or indirect regulation of CEBPA. So, it is still need to be explored further about the involvement of CEBPA in myeloid differentiation in CML. Many genes have to be examined, such as hnRNP-E2, UPF1, S100A9 etc., to confirm pathway that would be blocked in CML.

Other than hnRNP-E2, CEBPA was also regulated by other genes. In AML, calreticulin was proved as a one of the major genes that inhibit CEBPA translation. TRIB-2 could also induce the degradation of CEBPA protein. On the other hands, CEBPA protein interaction with AML1-ETO or c-jun was also known to inhibit the function of CEBPA itself [29-33]. It assumed that there are many factors that regulate the CEBPA expression and its activities in myeloid differentiation process. In CML, the genes that influence the expression and CEBPA activity were still unclear. Many factors still need to be examined to prove the role of CEBPA in CML progression. It is suggested that might be several marker need to be used as the progression marker for CML.

Conclusion

The mRNA expression level of Hes-1 in chronic phase was statistically higher than those in the late phase of CML, whereas the CEBPA mRNA expression level in chronic phase late phase did not statistically different.

Since many factors influence Hes-1 and CEBPA expression, further study need to be done to further explore Hes-1 and CEBPA as progression marker of CML. In the future study, the dynamic of Hes-1 and CEBPA expression level of the CML patients from chronic phase to blast crisis also need to be analysis, in order to get the picture of the role of those gene in the CML progression.

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