

## Detection of *CEBPA* mutation gene in acute myeloid leukemia patients

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### Abstract

**Background:** The mutations of CCAAT/enhancer binding protein-alpha (*CEBPA*) gene are evaluated as favorable prognostic tools for acute myeloid leukemia (AML) patients. The gold standard method for detection of *CEBPA* gene mutations is direct sequencing. This method has some disadvantages, and *CEBPA* mutations can occur across the whole gene, and there should be a screening test before designating the type of mutation by direct sequencing.

**Objective:** This study was to evaluate the ability of denaturing high-performance liquid chromatography (DHPLC) for screening *CEBPA* mutations.

**Method:** The coding region of *CEBPA* gene in 114 AML patients and 40 normal controls were screened by DHPLC and confirmed by direct sequencing.

**Results:** Our results demonstrated that DHPLC is a useful screening test to detect *CEBPA* gene mutations in AML patients. Fifteen types of *CEBPA* gene mutations including insertion, duplication, deletion, and substitution were also detected by DHPLC.

**Conclusion:** A combination of DHPLC and direct sequencing is an appropriate approach for detecting *CEBPA* mutations.

**Keywords:** *CEBPA*, Mutation, AML, DHPLC, Direct sequencing

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## Introduction

*CEBPA* or CCAAT/ enhancer binding protein alpha (C/EBP $\alpha$ ) gene is located on long arm of chromosome 19 at band q13.1. *CEBPA* is an intronless gene that encodes a member of leucine zipper transcription factor (CEBP $\alpha$ ) and it acts as an inhibitor of cell proliferation and tumor suppressor<sup>1-6</sup>. In hematopoietic system, *CEBPA* has an important role in myeloid lineage differentiation. It is specifically up-regulated in granulocytic differentiation and principally expressed in myelomonocytic cell<sup>3, 7-10</sup>. The *CEBPA* knockout mice results in lacking mature granulocytes, whereas the development of other hematopoietic lineages are present in normal proportion<sup>11</sup>. Therefore, it is assumed that *CEBPA* inactivation might be specific to differentiation block in myeloid lineage that is specific to AML<sup>12</sup>.

*CEBPA* mutation can occur across the entire gene and exhibits various patterns such as deletion, insertion, duplication and point mutation but is assemble in two main hotspots, N- and C-terminal domains. N-terminal out-of-frame insertions or deletions increase a dominant-negative p30 isoform. The imbalance between p42 and p30 isoform interferes cell cycle arrest and differentiation. Mutations in the C-terminal tend to destroy function of DNA binding and leucine zipper domains due to the insertion/deletion mutations in those domains. AML patients can have either one or two mutations. Two mutations are called double mutation. They involve N- and C-terminal alterations that are presumed to be biallelic pattern. However, homozygous mutation has been reported in AML cases<sup>2, 3, 13-16</sup>. Mutations of *CEBPA* gene were suggested to be a good prognostic factor in AML patients, especially in cytogenetically normal AML<sup>17</sup>. Several lines of evidence demonstrated that disease-free survival (DFS) and overall survival

(OS) are significantly longer in mutate-*CEBPA*<sup>18</sup>. Recent studies have inferred that the good prognosis may be restricted to double, not single *CEBPA* mutant-patients<sup>13, 14, 19</sup>.

Though the direct sequencing is a gold standard method for detection of *CEBPA* gene mutations, it is time-consuming, labor-intensive, expensive procedure and requires expertise to interpret results<sup>20</sup>. Moreover, *CEBPA* mutations can occur across the entire coding region of the gene and have various patterns of mutations. Hence, efficient screening test before identifying types of mutations by direct sequencing is necessary. Denaturing high-performance liquid chromatography (DHPLC) has been widely used method to detect any possible genetic alteration (insertions, deletions, duplications and point mutations/polymorphism)<sup>13</sup>. In addition, DHPLC has been described as a highly sensitive, simple, rapid, inexpensive and facile assay to interpret results<sup>21, 22</sup>. Therefore, the purpose of this study was to evaluate the ability of DHPLC method for screening *CEBPA* mutations and to assess an appropriateness of combination between DHPLC and direct sequencing for detecting *CEBPA* mutations in Thai AML patients.

## Materials and methods

### Samples

Bone marrow or peripheral blood samples from 114 AML patients were collected. The study was approved by Ramathibodi Hospital Ethic Committee (No.2011/371). A total of 40 samples of peripheral blood from healthy volunteers were normal control for mutations and distilled water was used as a negative control.

### *CEBPA* gene amplification

EDTA blood or bone marrow was extracted to prepare genomic DNA by using High Pure PCR

Template Preparation kit (Roche Diagnostics, USA). The entire coding region of *CEBPA* gene was amplified by polymerase chain reaction (PCR) with 3 overlapping primer pairs (Table 1). PCR reaction was performed with final reaction volume of 25  $\mu$ l containing 50 ng DNA, 0.25  $\mu$ M of each primer, 1.25U of AmpliTaq Gold polymerase, 200  $\mu$ M of each dNTP, 1xPCR buffer, 1.5 mM MgCl<sub>2</sub> and 5% of DMSO. The PCR program consisted of 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and final elongation at 72°C for 10 minutes.

#### Denaturing high-performance liquid chromatography (DHPLC)

5  $\mu$ l of PCR product from a patient was mixed with 5  $\mu$ l of PCR products from a normal control (wild type). Heteroduplex formation was performed as following condition: 94°C for 4 minutes for sample denatured and then slowly reannealed by reduce temperature at rate of 0.1°C/4

second until temperature down to 25°C in the Veriti 96 well Fast Thermal cycler (Applied Biosystem, USA). The samples were identified by DHPLC WAVE® system 4500 (Transgenomic® USA) at 67.8°C, 67.0°C and 66.5°C for fragment 1, 2 and 3, respectively.

#### Direct sequencing

In direct sequencing method, PCR products were purified by ExoSAP-IT Reagan. The purified products were sequenced using Big Dye Terminators with ABI Prism 3130 Genetic Analyzer (Applied Bio systems, USA). The sample sequences were compared with *CEBPA* genomic sequences (NM\_004364.3) using SeqScape Software version 2.5 (ABI).

## Results

We evaluated our approach by analyzing 114 AML patient samples and 40 samples of normal control by both DHPLC and direct sequencing. The results of DHPLC demonstrated that 76 AML

**Table 1** Sequences and properties of primers for *CEBPA* gene study

Primer name	Sequence 5'-3'	Position 5'-3' (relative to main translational start site)	Primer Length (bp)	Tm (°C)	%GC	Product size (bp)
1 Forward	TCGCCATGCCGG	81-104	24	62.8	58.3	
	GAGAACTCTAAC	(-29 - -6)				
1 Reverse	AGCTGCTTGGCT	607-628	21	61.0	54.5	548
	TCATCCTCCT	(497-518)				
2 Forward	TACCTGGACGGC	522-540	19	62.2	68.4	
	AGGCTGG	(412-430)				
2 Reverse	ACCCGGTACTCG	949-970	22	60.6	54.5	449
	TTGCTGTTCT	(839-860)				
3 Forward	GGCCCTGGCAGC	852-871	20	68.7	75.0	
	GCGCTCAA	(742-761)				
3 Reverse	CCCTCCTCGCAG	1394-1414	21	64.4	61.4	563
	GGAGAAGGCC	(1284-1304)				

patients and 33 normal controls presented wild type chromatogram with one single peak. Thirty-eight patient and seven normal samples showed more than one single peaks in one or more fragments, corresponding to genetic alteration (Figure 1). All samples were confirmed by direct sequencing assay and revealed that 76 samples were wild types, 28 samples were polymorphisms and 10 samples were mutations in AML patients. In addition, all 7 normal samples were confirmed as polymorphisms.

*CEBPA* variants were detected in 10 of 38 samples (26.3%). After confirmation by DNA sequencing, these 10 samples presented 15 distinct types of mutations (Table 2). Seven of ten samples had double mutations (patient No.1, 23, 46, 71, 77, 96, and 100) that consist of the mutation at N and C terminal region. Three remaining samples had a single mutation (patient No.50, 55, and 95). Most of the N-terminal mutations were out of frame insertion/duplication or out of frame deletion whereas most of the C-terminal mutations were inframe insertion/duplication or inframe deletion. The mutations of N-terminal cause n-terminal stop resulting in truncated protein (p30). The mutations of C-terminal cause abnormalities at bZIP leucine domain resulting in destruction of a binding to DNA and a dimerization.

Two types of polymorphisms were found in 28 samples whose mutations were previously reported. The c.584-589 dup ACCCGC was a 6

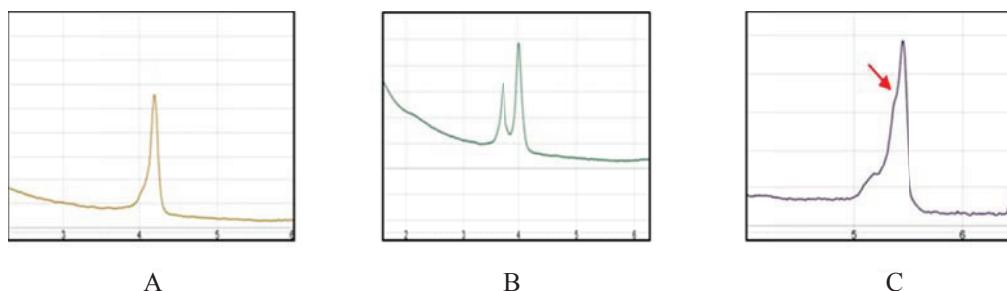
nucleotides in-frame duplication in transactivation domain 2 (P194\_H195dup) which was found in 23 samples. This polymorphism was also found in the mutated patient that presented double mutation. In addition, 2 of 23 samples were found as 12 nucleotides in frame duplication at this region (ACCCGC ACCCGC). Five samples were point polymorphism c.690 G>T (T230T). DHPLC chromatogram of this alteration showed relatively subtle changes (Figure 1C).

Seven samples of normal control showed more than one single peak in fragment 2 of DHPLC chromatogram. Results of direct sequencing revealed that all alterations were previously reported polymorphism. Four samples were c.584-589 dup ACCCGC (10%), while three samples were c.690 G>T (7.5%).

All types of *CEBPA* gene mutations including insertion, duplication, deletion and substitution were also detected by DHPLC (Figure 2). Our results indicated that DHPLC was an effective method for separation between nucleotide variants and wild type samples. Moreover, all mutations were detected and none was missed by this method.

## Discussion

*CEBPA* mutations were detected in 10 samples (8.8%). This incidence was less than that is the report from Southeast Asian AML patients (13.8%)<sup>23</sup>. The results from this study demonstrate



**Figure 1** DHPLC chromatogram of wild-type and polymorphism/mutation of *CEBPA* gene; wild-type (A) and polymorphism/mutation (B and C)

that *CEBPA* mutations are clustered in two hotspots: N-terminal and C-terminal. N-terminal domain consists of the region before TAD1, TAD1 and the region before TAD2 (amino acid at 1-120) while C-terminal domain consists bZIP region (amino acid at 278-358)<sup>13, 15</sup>. Most of the mutations at N-terminal

domain are frame shift insertions/duplication or deletions (c.185-189 del TCGAC, c.197-198 del CC, c.247del C, c.326-327 ins 26 bp, c.229-230 ins CAGGA, c.211-251 del 41 bp). For C-terminal domain most of the mutations are in-frame insertions/duplication or deletions mutation (c.904-921 dup 18

**Table 2** Types of *CEBPA* mutations and polymorphism along with predicted amino acid change of *CEBPA* gene

No. of patient	Nucleotide change <sup>a</sup>	Amino acid change <sup>b</sup>
<b>Double mutations</b>		
No.1	c.326-327 ins GGGCGCGCCCGCGACTTGACTACCC c.921-938 del AACGTGGAGACGCAGCAG	G114fsX168 N307_Q312delINVETQQ
No.23	c.197-198 del CC c.934-936 dup CAG	A66fsX106 Q312dup
No.46	c.197-198 del CC c.934-936 dup CAG	A66fsX106 Q312dup
No.71	c.185-189 del TCGAC c.904-921 dup AAGGCCAAGCAGCGCAAC	I62fsX105 K302_N307dupKAKQRN
No.77	c.247del C c.923-925 dup TGG	Q83fsX159 V308dup
No.96	c.229-230 ins CAGGA c.946-947 ins TGG	F77fsX161 L315_E316insV
No.100	c.211-251 del GCCGCC TTCAACGACGAGTTCTGGCCGACCTGTTCCAGCA c.937-939 dup AAG	A71fsX93 K313dup
<b>Single mutation</b>		
No.50	c.268 A>T	K90X
No.55	c.929 C>G and c.929-930 ins CAG	T310S and T310_Q311insR
No.95	c.929ins C	Q311fsX320

a: description at the cDNA level (reference sequence: NM\_004364.3) The major translational start codon at position 111 in that reference sequence was renumbered to start at position 1

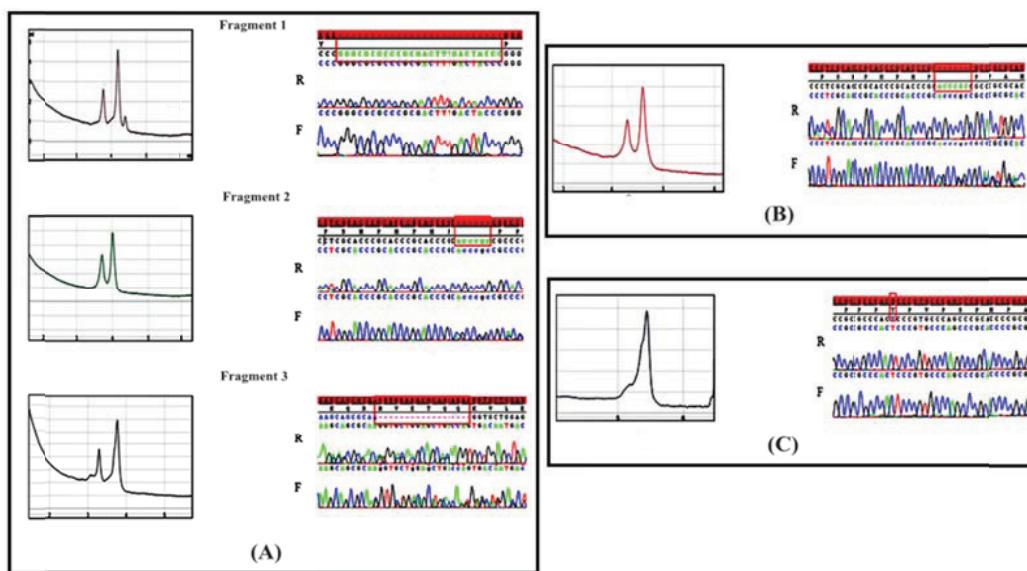
b: description at the protein level (NP\_004355.2)

bp, c.921-938 del 18 bp, c.923-925 dup TGG, c.934-936 dup CAG, c.929-930 ins CAG, c.946-947 ins TGG, c.937-939 dup AAG). These results are similar to studies of Green et al, Preudhomme et al and Fuster et al<sup>13, 16, 24</sup>. Moreover, in TAD 2 there was no mutation reported but c.584-589 dup ACCCGC polymorphism was present. Therefore, mutations in this region are uncommon<sup>24</sup>. In addition, 7 of 10 samples with double mutations consist of mutation at N and C terminal. Three remaining samples had a single mutation. This was the same result as reported previously<sup>15</sup>.

The c.584-589 dup ACCCGC and c.690 G>T are two types of polymorphisms previously detected<sup>23, 25, 26</sup>. Twenty-three of 114 patient samples were c.584-589 dup ACCCGC (20.2%), while five samples were c.690 G>T (4.4%). The frequency was similar to the study in Southeast Asian AML patients (20.2% and 3.6%, respectively)<sup>23</sup>. Some studies reported that c.584-589 dup ACCCGC was mutation<sup>27</sup>. Since then this alteration was reported as a polymorphism and it was not associated with

pathogenic status<sup>28-30</sup>. Why it is the reason that is necessary to separate mutation from polymorphism. In addition, we found 12 nucleotides in-frame duplication at TAD2 region (ACCCGC ACCCGC) which was reported to be polymorphism<sup>31</sup>. Furthermore, our results of patients with double mutations also demonstrate polymorphism (c.584-589 dup ACCCGC) as reported previously<sup>24, 32</sup>. This polymorphism may be associated with the acquisition of additional mutations which requires confirmation<sup>32</sup>.

The fragment analysis method based in PCR capillary electrophoresis is a popular screening method in prior studies<sup>20, 24, 26, 31, 33, 34</sup>. However, this method is based on an alteration in fragment size, so it cannot detect substitution or point mutation/polymorphism, while DHPLC can detect possible mutation or polymorphism (point, insertion, duplication or deletion)<sup>13</sup>. The comparison between DHPLC and direct sequencing demonstrated an efficacy of DHPLC method to separate mutation and polymorphism from wild type for 100% (38/38). However, this method still requires direct sequencing



**Figure 2** DHPLC chromatograms and partial sequences of patient No.1 demonstrated insertion in fragment 1, deletion in fragment 3 and polymorphism in fragment 2 (A). Polymorphism in fragment 2 (B) and (C) can be detected in normal control.

method for separation of mutation or polymorphism samples, and also to designate mutation types and polymorphism types. Sometimes, DHPLC chromatograms of some samples were difficult to interpret such as point polymorphism (690G>T), therefore, sequencing method is needed to confirm such result<sup>35</sup>. In conclusion, DHPLC is a highly sensitive, reliable, and rapid diagnostic test that

detects *CEBPA* mutations as well as direct sequencing. However, DHPLC may need to be performed in concert with sequencing to unravel the significance of abnormal chromatograms that certainly indicate nucleotide variations and possibly sporadic new mutations.

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## การตรวจสอบการกลายของยีน CEBPA ในผู้ป่วยโรคเม็ดโลหิตขาวชนิดมัยอิลloydแบบเจียบพลันมีประ予以ชันสำหรับการพยากรณ์โรคไปในแนวทางที่ดี วิธีมาร์ฐานสำหรับตรวจทางการกล่ายของยีน CEBPA คือการหาลำดับเบส แต่วิธีนี้มีข้อจำกัดหลายประการ อีกทั้งการกล่ายของยีน CEBPA สามารถเกิดได้ทุกตำแหน่งของยีน ดังนั้นควรมีการตรวจคัดกรองก่อนที่จะตรวจยืนยันและบ่งชี้ชนิดของการกลายด้วยวิธีการหาลำดับเบส

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### บทคัดย่อ

**ความเป็นมา:** การกลายของยีน CCAAT/enhancer binding protein-alpha (CEBPA) ในผู้ป่วยโรคเม็ดโลหิตขาวชนิดมัยอิลloydแบบเจียบพลันมีประ予以ชันสำหรับการพยากรณ์โรคไปในแนวทางที่ดี วิธีมาร์ฐานสำหรับตรวจทางการกล่ายของยีน CEBPA คือการหาลำดับเบส แต่วิธีนี้มีข้อจำกัดหลายประการ อีกทั้งการกล่ายของยีน CEBPA สามารถเกิดได้ทุกตำแหน่งของยีน ดังนั้นควรมีการตรวจคัดกรองก่อนที่จะตรวจยืนยันและบ่งชี้ชนิดของการกลายด้วยวิธีการหาลำดับเบส

**วัตถุประสงค์:** เพื่อประเมินความสามารถของวิธีดีเอชพีเอลซีในการตรวจคัดกรองสำหรับทางการกล่ายของยีน CEBPA

**วิธีการศึกษา:** โดยทำการศึกษาการกล่ายของยีน CEBPA จากผู้ป่วยโรคเม็ดโลหิตขาวชนิดมัยอิลloydแบบเจียบพลันจำนวน 114 ตัวอย่าง และจากของคนปกติจำนวน 40 ตัวอย่าง ด้วยวิธีดีเอชพีเอลซีควบคู่กับการหาลำดับเบส

**ผลการศึกษา:** วิธีดีเอชพีเอลซีมีประสิทธิภาพในการแยกตัวอย่างที่ผิดปกติออกจากตัวอย่างปกติ โดยที่วิธีดีเอชพีเอลซียังสามารถตรวจจับรูปแบบการกล่ายของยีน CEBPA ได้ทั้ง 15 รูปแบบ ซึ่งประกอบด้วยการกล่ายแบบ insertion, duplication, deletion, and substitution

**สรุป:** วิธีดีเอชพีเอลซีเป็นวิธีที่มีประสิทธิภาพในการตรวจคัดกรองการกล่ายของยีน CEBPA ในผู้ป่วย AML และวิธีดีเอชพีเอลซีร่วมกับวิธีการหาลำดับเบสเป็นวิธีการที่เหมาะสมในการตรวจทางการกล่ายของยีน CEBPA

**คำสำคัญ:** CEBPA, Mutation, AML, DHPLC, Direct sequencing

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