

การใช้ดีเอ็นเอซีพีแอลซีสำหรับตรวจคัดกรองการกลายของยีน *CEBPA* ในผู้ป่วยโรคมะเร็งเม็ดโลหิตขาวชนิดมัยอีลอยด์แบบเฉียบพลัน

The Use of DHPLC for Mutation Screening of *CEBPA* Gene in Acute Myeloid Leukemia

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บทคัดย่อ

การกลายของยีน CCAAT/enhancer binding protein-alpha (*CEBPA*) ในผู้ป่วยโรคมะเร็งเม็ดโลหิตขาวชนิดมัยอีลอยด์แบบเฉียบพลันมีประโยชน์สำหรับการพยากรณ์โรคไปในแนวทางที่ดี วิธีมาตรฐานสำหรับตรวจหาการกลายของยีน *CEBPA* คือการหาลำดับเบส แต่วิธีนี้มีข้อจำกัดหลายประการ อีกทั้งการกลายของยีน *CEBPA* สามารถเกิดได้ทุกตำแหน่งของยีน ดังนั้นควรมีการตรวจคัดกรองก่อนที่จะตรวจยืนยันและบ่งชี้ชนิดของการกลายด้วยวิธีการหาลำดับเบส โดยวิธีที่ใช้ตรวจคัดกรองการกลายของยีน *CEBPA* ต้องเป็นวิธีที่มีความถูกต้อง รวดเร็วและปฏิบัติได้ง่าย วัตถุประสงค์ของการศึกษาค้นคว้าครั้งนี้เพื่อประเมินความสามารถของวิธีดีเอ็นเอซีพีแอลซีในการตรวจคัดกรองสำหรับหาการกลายของยีน *CEBPA* โดยทำการศึกษากการกลายของยีน *CEBPA* ในผู้ป่วยโรคมะเร็งเม็ดโลหิตขาวชนิดมัยอีลอยด์แบบเฉียบพลันจำนวน 40 ราย ด้วยวิธีดีเอ็นเอซีพีแอลซีควบคู่กับการหาลำดับเบส จากผลการวิจัยพบว่าวิธีดีเอ็นเอซีพีแอลซีเป็นวิธีตรวจคัดกรองที่ดี และมีประโยชน์ในการตรวจวิเคราะห์การกลายของยีน *CEBPA* ในผู้ป่วยโรคมะเร็งเม็ดโลหิตขาวชนิดมัยอีลอยด์แบบเฉียบพลัน

ABSTRACT

The mutations of CCAAT/enhancer binding protein-alpha (*CEBPA*) gene are evaluated as a favorable prognostic tool for AML patients. The gold standard method for detection of *CEBPA* gene mutations is direct sequencing. However, this method has some disadvantages and *CEBPA* mutations can occur across the whole gene. Thus, it should have a screening test before designating the type of mutation by direct sequencing. This screening method requires an accurate, fast and easy method. The purpose of this study was to evaluate the ability of denaturing high-performance liquid chromatography (DHPLC) method for screening *CEBPA* mutations. The coding region of *CEBPA* gene in 40 AML patients was screened by DHPLC and confirmed by direct sequencing. Our results suggested that DHPLC was a useful screening test to detect *CEBPA* gene mutations in AML patients.

คำสำคัญ: *CEBPA*, การกลาย, มะเร็งเม็ดโลหิตขาวชนิดมัยอีลอยด์แบบเฉียบพลัน, ดีเอ็นเอซีพีแอลซี, คัดกรอง

Keywords: *CEBPA*, mutation, acute myeloid leukemia, DHPLC, screening

INTRODUCTION

CEBPA or CCAAT / enhancer binding protein alpha gene locates on chromosome 19q13.1. It is an intronless gene which encodes a transcription factor protein (C/EBP α). C/EBP α is an importance transcription factor in myeloid differentiation and specifically expressed in myeloid lineage. The *CEBPA* knockout mice present lacking mature granulocytes, whereas, the development of other hematopoietic lineages shows normal proportion. Therefore, it has been assumed that *CEBPA* inactivation might contribute to the differentiation block specific to AML (Koschmieder *et al.*, 2009).

The mutation of *CEBPA* can occur throughout the entire gene and various patterns such as deletion, insertion, duplication and point mutation but most mutation are found in two hotspots, N- and C- terminal domains. Patients can have two mutations (involves N- plus C-terminal variant) are called double mutations that are assumed to be biallelic pattern (Green *et al.*, 2010). Mutations of *CEBPA* gene were suggested to be a good prognostic factor in AML patients, especially in cytogenetically normal AML (Marcucci *et al.*, 2008). Several lines of evidence suggested that overall survival (OS) and disease-free survival (DFS) was significantly longer in AML patients with *CEBPA* mutations. Some studies show that this advantage is only present in patients with double mutations. This advantage may be results of double mutations which have a specific gene expression pattern, while single mutations and wild type do not. Moreover, patients with double mutations usually show lower mutation in FLT3-ITD that is associated poor prognosis (Paz-Priel and Friedman, 2011). Previous reports performed *CEBPA* mutation detection as a real-time monitoring marker in AML patients at the relapse period (Smith *et al.*, 2006; Tiesmeier *et al.*, 2003).

Though, the direct sequencing is a gold standard method for detection of *CEBPA* gene mutations but it is time-consuming, labor-intensive, expensive procedure and requires expertise to interpret results (Ahn *et al.*, 2009). Previously mentioned, *CEBPA* mutations can occur across the entire coding region of the gene and have various patterns of mutation. Hence, efficient screening test before identifying types of mutations by direct sequencing is necessary. Denaturing high-performance liquid chromatography (DHPLC) is an interesting method to detect any possible genetic alteration (insertions, deletions, duplications and point mutations/polymorphism) (Green *et al.*, 2010). Moreover, DHPLC has been described as a highly sensitive, simple, rapid, inexpensive and facile assay to interpret results (Xiao and Oefner, 2001). In this study, the coding region of *CEBPA* gene was studied in 40 AML patients by DHPLC to screen *CEBPA* mutations. Finally direct sequencing method was done to confirm the results. Our data demonstrated that DHPLC method successfully distinguished wild-type and polymorphism/mutation in the patients. Therefore, we suggested that DHPLC was a useful screening test to detect *CEBPA* gene mutations in AML patients.

MATERIALS AND METHODS

The total of 40 samples of bone marrow or peripheral blood from AML patients was obtained. All samples were leftover specimen of routine assay in Human Genetics Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital. The genomic DNA was extracted by using High Pure PCR Template Preparation kits (Roche Diagnostics, USA). The quantity and quality of DNA were evaluated with spectrophotometer. The entire coding region of *CEBPA* gene was amplified by PCR using 3 pairs of overlapping primers. The PCR products were analyzed by DHPLC using mutation detection program (WAVE[®] system 4500, Transgenomic[®] USA) and confirmed by direct sequencing in both directions. The number of nucleotides was according to Genbank Accession No. NM_004364.3.

RESULTS AND DISCUSSION

In this study, 40 samples from AML patients were screened for *CEBPA* mutations using DHPLC. The results demonstrated that 30 samples presented wild-type chromatogram with one single peak (Figure 1A). Ten samples showed more than one single peaks in one or more fragments, corresponding to genetic alteration (Figure 1B). All samples were confirmed by direct sequencing assay and revealed that 30 samples were wild-type, 7 samples were polymorphism and 3 samples were mutation. These 3 samples presented 5 distinct types of nucleotide variants (c.185-189 del TCGAC, c.904-921 dup AAGCCAAGCAGCGCAAC, c.247del C, c.923-925 dup TGG and c.268 A>T). Two of three samples presented double mutations. All types of *CEBPA* gene mutations (insertion, duplication, deletion and substitution) were also detected by DHPLC. The sensitivity of DHPLC method to detect the numbers of *CEBPA* gene mutations is 100% (5/5). Therefore, this result suggested that DHPLC was an effective method for separating between variant positive and normal samples. However, it still requires direct sequencing method for designating mutation types and polymorphism (Figure 2).

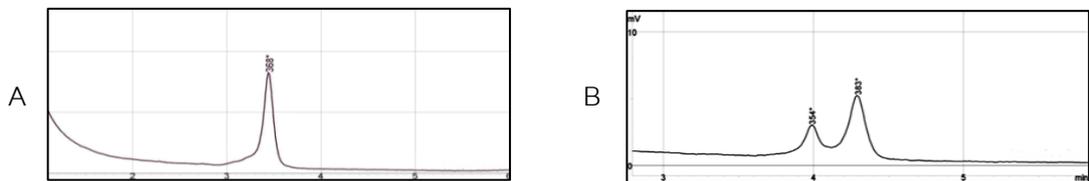


Figure 1 DHPLC chromatogram of *CEBPA* gene: wild-type (A) and polymorphism/mutation (B).

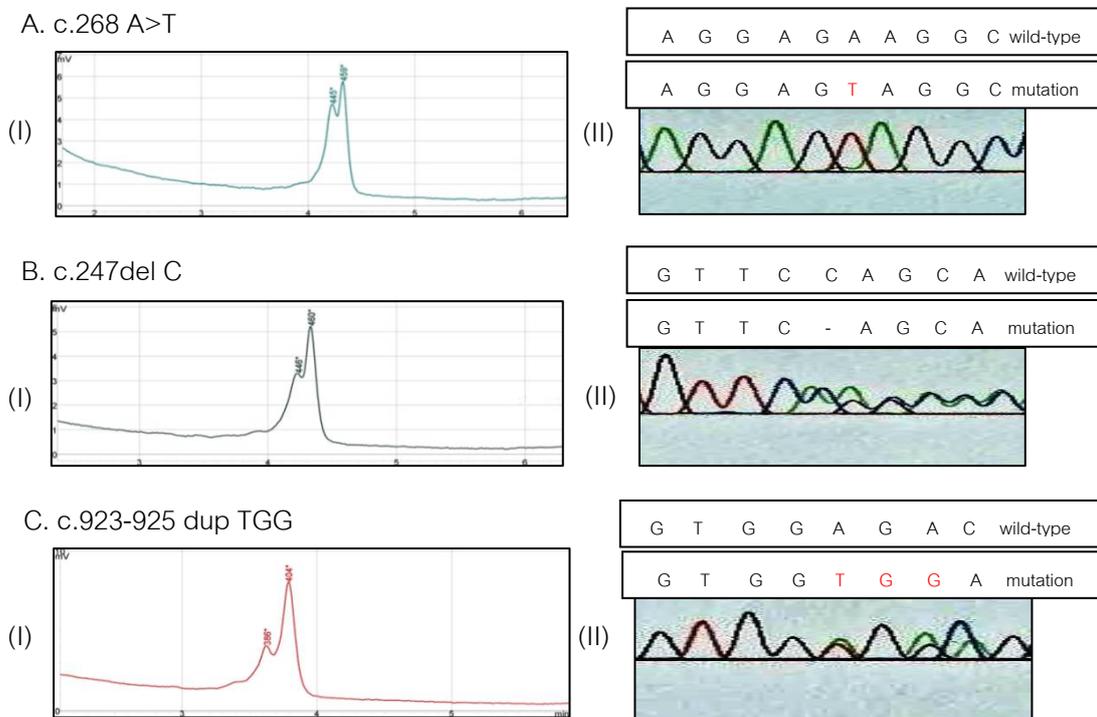


Figure 2 Examples of DHPLC chromatograms and partial sequences of *CEBPA* gene mutation in AML patients.

CEBPA mutations were detected in 3 samples (7.5%). The frequency was comparable to that of The United States of America (7.3%) (Pabst *et al.*, 2001), United Kingdom (7%) (Green *et al.*, 2010) and Germany (7.7%) (Tiesmeier *et al.*, 2003). The mutations were detected in 2 hotspots. N-terminal mutations were frameshift insertion/deletion mutations while in-frame duplication/deletion mutations were detected in C-terminal (Leroy *et al.*, 2005). One type of polymorphisms detected in TAD2 region was shown in 7 of all patients (17.5%). The c.584-589 dup ACCCGC was a 6 nucleotide in-frame duplication polymorphism (P194_H195dup). It was previously described to be a polymorphism (Wouters *et al.*, 2007).

CONCLUSION

Our results demonstrated that DHPLC is a useful screening test to detect *CEBPA* gene mutations in AML patients. Combination of DHPLC and direct sequencing is an appropriate approach for detecting *CEBPA* mutations.

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REFERENCES

- Ahn JY, Seo K, Weinberg O, Boyd SD, Arber DA. A comparison of two methods for screening *CEBPA* mutations in patients with acute myeloid leukemia. *J Mol Diagn.* 2009 Jul;11(4):319-23.
- Green CL, Koo KK, Hills RK, Burnett AK, Linch DC, Gale RE. Prognostic significance of *CEBPA* mutations in a large cohort of younger adult patients with acute myeloid leukemia: impact of double *CEBPA* mutations and the interaction with *FLT3* and *NPM1* mutations. *J Clin Oncol.* 2010;28(16):2739-47.
- Koschmieder S, Halmos B, Levantini E, Tenen DG. Dysregulation of the *C/EBPalpha* differentiation pathway in human cancer. *J Clin Oncol.* 2009;27(4):619-28.
- Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C. *CEBPA* point mutations in hematological malignancies. *Leukemia.* 2005;19(3):329-34.
- Marcucci G, Maharry K, Radmacher MD, Mrozek K, Vukosavljevic T, Paschka P, et al. Prognostic significance of, and gene and microRNA expression signatures associated with, *CEBPA* mutations in cytogenetically normal acute myeloid leukemia with high-risk molecular features: a Cancer and Leukemia Group B Study. *J Clin Oncol.* 2008;26(31):5078-87.
- Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, et al. Dominant-negative mutations of *CEBPA*, encoding CCAAT/enhancer binding protein- α (*C/EBPalpha*), in acute myeloid leukemia. *Nat Genet.* 2001;27(3):263-70.
- Paz-Priel I, Friedman A. *C/EBPalpha* dysregulation in AML and ALL. *Crit Rev Oncog.* 2011;16(1-2):93-102.
- Smith L-L, Pearce D, Smith ML, Jenner M, Andrew Lister T, Bonnet D, et al. Development of a quantitative real-time polymerase chain reaction method for monitoring *CEBPA* mutations in normal karyotype acute myeloid leukaemia. *British Journal of Haematology.* 2006;133(1):103-5.
- Tiesmeier J, Czwalinna A, Müller-Tidow C, Krauter J, Serve H, Heil G, et al. Evidence for allelic evolution of *C/EBPalpha* mutations in acute myeloid leukaemia. *British Journal of Haematology.* 2003;123(3):413-9.
- Wouters BJ, Louwers I, Valk PJ, Lowenberg B, Delwel R. A recurrent in-frame insertion in a *CEBPA* transactivation domain is a polymorphism rather than a mutation that does not affect gene expression profiling-based clustering of AML. *Blood.* 2007;109(1):389-90.
- Xiao W, Oefner PJ. Denaturing high-performance liquid chromatography: A review. *Hum Mutat.* 2001;17(6):439-74.