Establishment of a Detecting Procedure and Analysis of CEBPA-TAD Genetic Mutation in Vietnamese Acute Myeloid Leukemia Patients

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Abstract: Acute myeloid leukemia (AML) is caused by mutations leading to the loss of control over the proliferation and differentiation of leukocytes in the bone marrow. In Vietnam, studies focusing on the statistics, types, rate and molecular characteristics of common genetic mutations in AML patients are still limited. Identification of chromosomal mutation using the standard karyotyping techniques has assisted effectively AML diagnosis, however, in approximate 45% of AML cases, karyotyping analysis show normal cytogenesis due to abnormalities occurring at the molecular level. In this study, we focused on the establishment of a procedure to detect CCATT-enhancer binding protein α mutations in Transactivation domain region (CEBPA-TAD), which are considered poor prognostic factor for treatment. Using this procedure, one sample carrying a 17-nucleotide deletion in TAD1 domain resulting in a frameshift and a premature stop, was identified and confirmed by sequencing. From these results, we aim to continue screening a larger sample size to get more significant statistical data and investigate the correlation of CEBPA mutation status with clinical characteristic to assist prognosis and treatment.

Keywords: Acute myeloid leukemia (AML), CCAAT enhancer binding protein α (CEBPA), mutation.

1. Introduction

Leukemia are hematological malignancies of leukocytes or their progenitors, and are divided into four main groups: Acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoid leukemia (ALL) and chronic lymphoid leukemia (CLL). AML occurs in both children and adults and accounts for 54.6% of all leukemia cases. Currently, genetic analysis methods using karyotyping techniques to detect genetic abnormalities have a very important role in the diagnosis, prognosis and treatment in AML patients. However, in approximately 45% of AML cases, karyotyping results show normal cytogenesis (AML-NK – normal karyotyping) due to abnormalities occurring at the molecular level instead of cellular level [1 - 3]. There are many genetic mutations associated with AML, highly concentrated on three genes: Nucleophosmin 1 (NPM1), FMS-like tyrosine kinase (FLT3), and CCAAT enhancer binding protein α (CEBPA).
kinase 3 (FLT3), and CCAAT / enhancer binding protein alpha (CEBPA) with a total frequency of 80% [3, 4]. CEBPA mutations were reported to account for 10-15% of all AML-NK cases, seriously affecting proliferation and differentiation of myeloid cells.

CCAAT enhancer binding protein α (C/EBPα) is a transcription factor coded by the intronless gene CEBPA, found on the long arm of chromosome 19 at 19q13.1 [5, 6]. C/EBPα protein consists of a basic region and a leucine zipper domain in the C terminus and two transactivation domains, TAD1 and TAD2, in the N terminus [4]. There are two main isoforms of C/EBPα: the full-length 42 kDa protein (p42) and the truncated 30 kDa protein (p30). The p30 isoform is translated from an internal start site in the mRNA and the protein lacks the first 119 amino acids of the N terminus, which includes TAD1. It has been found that the p30 protein has a lower transcriptional activation potential than the p42 protein [7]. The majority of characterized mutations in CEBPA included dominantly length mutations and substitutions mutations in TAD region of the N terminus and bZIP region of the C terminus.

There are various techniques to detect CEBPA-TAD mutations such as electrophoresis and direct sequencing. However, a rapid and well established diagnostic procedure has not yet been set up for regular use in hospitals in Vietnam. Therefore, the objective of this study is to establish a procedure to detect CEBPA-TAD mutations, preliminarily screen, analyse and characterize the mutations in laboratory, then transfer to medical facilities.

2. Materials and methods

2.1. Blood samples

Blood specimens of patients diagnosed with Acute Myeloid Leukemia (AML) were collected from National Institute of Hematology and Blood Transfusion (NIHBT) following the guidelines and ethical rules of NIHBT. DNA extraction was done with Magpure Genomic DNA nano kit (AnaBio). The isolated DNA was examined by electrophoresis on 1% agarose gel and preserved at -20°C.

2.2. CEBPA-TAD fragment amplification

In order to detect CEBPA-TAD mutations, a DNA fragment of 447 bp was amplified using CEBPA forward 5’-GGAGAACTCTAACTCCCCATGG-3’ and CEBPA reverse 5’-AGCCCTGCCGTCCAGGTAGC-3’. The components of the PCR reaction were optimized to achieve the best efficiency. PCR mixture (25µl total volume) included 2.5µl of 10X Dream Taq Buffer, 0.2 mM for each deoxynucleotide triphosphate, 10 pM for each primer, 0.5 unit of Dream Taq DNA polymerase (Thermo Scientific), 5% DMSO and 20-50 ng genomic DNA. The PCR mixture was denatured at 94°C for 5 minutes; run for 35 cycles of 94°C for 1 minute, 60°C for 45 seconds, 72°C for 1 minute, and finally extended at 72°C for 15 minutes.

2.3. Detecting CEBPA-TAD mutations by gel electrophoresis

PCR products were separated by electrophoresis on 5% nondenaturing polyacrylamide gel in 0.5X TBE buffer and 3% Ultraphor™ agarose gel in 1X TBE buffer. Then, the gel was stained by ethidium bromide solution (1 µg/ml) for 5-10 minutes, visualized and photographed by Bio-Rad Gel Doc system.

2.4. Cloning and sequencing of CEBPA-TAD mutant

The samples were separated by electrophoresis on 3% Ultraphor™ agarose gel. After the gel was stained with ethidium bromide and exposed under UV, the suspected
mutant band was cut out of the gel and purified by Wizard®-SV gel purification kit (Promega) according to manufacturer's instructions. DNA was dissolved in water and stored at -20°C.

Ligation reaction mixture of 10µl total volume contained: 5µl of 2X T4 ligase buffer, 25 ng of pGEMT-easy vector, 1.5 unit of T4 ligase and 5-10ng of purified DNA, and was incubated at 4°C overnight. The ligation mixture was used to transform Escherichia coli DH5α competent cells by heat shock method. The cells were then grown on TSA or TSB plates which were supplemented with 100µg/ml ampicillin, 40µl of 20mg/ml X-Gal, 40µl of 100mM isopropyl – D – galactoside (IPTG), in the incubator (37°C) overnight. The successful transformants (white colonies) from plates were selected and the presence of recombinant plasmid in colonies was verified through direct PCR with pUC19 primers (pUC19 forward 5’-GCTGCAAGGCGATTAAGTTG-3’ and pUC19 reverse 5’-GTTGTGTGAATTGTCACG-3’) and CEBPA-TAD primers. The positive colonies were grown in LB medium and the plasmid was extracted by QIaprep Spin Miniprep Kit (QIAgen). The purified plasmids carried the mutant DNA were sent for sequencing at 1st BASE (Malaysia).

3. Results and discussion

3.1. PCR reaction optimization to detect CEBPA-TAD mutations in AML patients

PCR is one of the most important steps of the screening process to detect CEBPA-TAD mutations. To obtain single, specific bands, both PCR components and thermal cycling conditions were optimized.

Template amount was calculated through images of isolated DNA on 1% agarose gel by comparing with 1kb laddering using ImageJ software. The results indicated that DNA concentration between 18-276 ng/µl were suitable.

![Diagram of CEBPA primer pairs](http://cancer.sanger.ac.uk/cosmic). CEBPA is rich in GC contents, thus it is difficult to specifically amplify a gene fragment, therefore, initially, PCR gave multiple bands and there were samples without any band (Figure 2A). To optimize amplification reaction, DMSO, a common PCR additive known to enhance specific amplification [8], was tested at three concentrations (0%, 3%, 5%) in combination with five different annealing temperatures (57°C-62°C, 1°C interval). Comparison of the results of different PCR condition combinations on gel electrophoresis indicated that 5% DMSO and annealing temperature at 60°C was the optimal condition because it gave the highest band intensity (Figure 2B). With these changes, the 447bp bands were able to be amplified with every sample (Figure 2C), thus, these conditions were used for all further experiments.
3.2. Establishment of the procedure for CEBPA-TAD mutation detection

Two different electrophoresis gels, vertical 5% polyacrylamide and horizontal 3% Ultraphor™ agarose were used to screen CEBPA-TAD mutations. The mutation samples showed a single band with shadows on 5% polyacrylamide because the separation between wild type and mutation bands was insufficient. Meanwhile, these two bands were obviously distinguishable on Ultraphor™ agarose gel which was a high resolution, matrix being able to separate fragments with 2% difference in size (Figure 3A). In summary, the procedure to screen and detect CEBPA-TAD mutation was established including three main steps: (1) extraction of DNA from blood of AML patients, (2) amplification of CEBPA-TAD fragment by specific primers, (3) electrophoresis using 5% polyacrylamide or 3% Ultraphor™ agarose gels to detect mutations depending on rapid screening or molecular characterization purposes, respectively. The total time for the procedure was approximately 4.5 hours (Figure 3B). In addition, cloning and sequencing steps could be done to further determine the mutant sequence.
reported to account for 93.5% of all of the N-terminal mutations [9]. Therefore, this PCR-based procedure accompanied with electrophoresis was chosen to detect CEBPA-TAD mutations.

3.3. Analysis of CEBPA-TAD mutations

Using this procedure to detect mutations in collected AML samples, one sample containing a 17-nucleotide deletion in CEBPA/TAD was found, which after conversion to aa sequence, was predicted to result in a frameshift in the position from nucleotide 379-395, in the TAD1 region (Figure 4A). This was a quite large deletion compared to previous studies, which reported the deletion mutations in the CEBPA-TAD region mostly are in the range between 1 and 15 nucleotides, consistently located in TAD1 [9].

Protein encoded by normal CEBPA gene contains 358 amino acids, whereas, only 100 amino acids remained in the mutated one found in this study due to the introduction of a premature stop (Figure 4B). The secondary structure of wild type and mutant proteins were predicted based on their polypeptide sequences and analyzed by (PS)2v2 (http://ps2.life.nctu.edu.tw). The results showed that the original structure of the α-helix was completely changed. The protein encoded by mutant gene had a partial of the α-helix structure, and most had distorted structure. As a result, this protein might lose not only its LZD (leucine zipper domain) together with DBD (DNA binding domain) region to interact
with DNA, but also its own function, possibly leading to poor prognostic factor for treatment (Figure 4C).

4. Conclusion

In this study, we successfully established a diagnostic procedure which could rapidly detect CEBPA-TAD mutations in AML patients in 4 – 4.5 hours. Moreover, using this procedure combined with cloning and sequencing, one mutation was identified which was a 17-nucleotide long deletion. In the future, this procedure can be applied in hospitals and clinics to support diagnosis and treatment of AML.

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References


Thiết lập quy trình để phát hiện phân tích đột biến trên vùng TAD của gen CEBPA từ các bệnh nhân ung thư bạch cầu dòng tủy cấp tính ở Việt Nam

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Tóm tắt: Ung thư bạch cầu cấp dòng tủy (AML) là một dạng ung thư máu gây ra bởi sự mất kiểm soát trong quá trình tăng sinh và biệt hóa tế bào bạch cầu trong tủy xương. Hiện nay, ở Việt Nam, các nghiên cứu về số lượng, tỷ lệ, các đột biến liên và đặc điểm phân tích của đột biến vẫn còn hạn chế. Phương pháp chẩn đoán mới đã phát hiện đột biến có thể 45% tổng số các bệnh AML có kiểu hình nhiễm sắc thể bình thường do người nhận gây bệnh ở cấp độ phân tử. Trong nghiên cứu này, chúng tôi tập trung vào việc xác định quy trình phân tích các đột biến vùng TAD trên gen CEBPA (CCATT enhancer binding protein α) - là logic đột biến được cho là có tiền lượng xâu trong quá trình điều trị ở bệnh nhân ung thư bạch cầu cấp dòng tủy. Sau khi áp dụng quy trình trên một số bệnh nhân, nghiên cứu đã tìm ra 1 đột biến mất đoạn 17 nucleotide vùng TAD1 gây ra đột biến kích thước làm thay đổi toàn bộ axit amin trong chuỗi polypeptit do gen mà hóa từ sau vị trí đột biến; đặc biệt hơn nữa là việc xuất hiện mà kết thúc sớm. Với kết quả đạt được, chúng tôi sẽ tiếp tục sử dụng quy trình để sàng lọc trên số lượng mẫu lớn hơn nhằm thu được kết quả thống kê đáng tin cậy về tần suất xuất hiện cũng như đặc điểm phân tử của các đột biến CEBPA-TAD ở bệnh nhân ung thư bạch cầu cấp dòng tủy Việt Nam nhằm hỗ trợ chẩn đoán và điều trị bệnh.

Từ khóa: Acute myeloid leukemia (AML), CCAAT enhancer binding protein α (CEBPA), đột biến.