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Research paper

Monitoring of clonal evolution of double *C-KIT* exon 17 mutations by Droplet Digital PCR in patients with core-binding factor acute myeloid leukemia

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ABSTRACT

C-KIT gene mutations result in the constitutive activation of tyrosine kinase activity, and greatly affect the pathogenesis and prognosis of core-binding factor acute myeloid leukemia (CBF-AML). *C-KIT* mutations are often found as single point mutations. However, the rate of double mutations has recently increased in AML patients. In this study, we detected six cases (18.8%) harboring double *C-KIT* exon17 mutations in 75 patients with CBF-AML. The clone composition and dynamic evolution were analyzed by sequencing and droplet digital PCR (ddPCR). Results revealed that these double mutations can be occurred in either the same or different clones. Different clones of double mutations may result in different sensitivity to the treatment of CBF-AML. The clones with N822 mutation responded better to treatment as compared to those with D816 mutation. Moreover, D816 clone was readily transformed into a predominant clone at relapse. Meanwhile, the predominant clones in the same patient may change during the progression of disease. The emerging mutation can originate from a small quantity of clones at diagnosis or newly acquired during the course of disease. Furthermore, patients with double mutations had better overall survival (OS) and event-free survival (EFS) than those with single mutation, but the differences did not reach statistical significance ($P > 0.05$). The ddPCR is an effective method for monitoring clonal evolution in patients with CBF-AML.

1. Introduction

Core-binding factor acute myeloid leukemia (CBF-AML) is characterized by t(8;21)(q22;q22) and inv(16)(p13.1q22)/t(16;16)(p13.1;q22) chromosomal aberrations [1]. CBF-AML is belongs to an AML group with a highly favorable prognosis. However, the risk and prognosis of CBF-AML patients may vary depending on the occurrence of genetic mutations, as exemplified by the mutation of *FLT3*, *C-KIT,* or *NRAS* gene [2]. *C-KIT* is a proto-oncogene located on chromosome band 4q11-12, which encodes a type III receptor tyrosine kinase [3]. *C-KIT* mutations may result in constitutive activation of the tyrosine kinase activity, which are considered to be the primary oncogenic event for the development of these tumors Generally, *C-KIT* mutation is associated with a higher incidence of relapse in CBF-AML and is classified into the intermediate risk group by NCCN Guideline [4–6]. These mutations occur most frequently in exon 17 (54%) encoding the kinase-activation loop, and in-frame insertions or deletions in exon 8 (28%) affecting the extracellular portion of KIT receptor [5]. Recently, tyrosine kinase inhibitors (TKIs) have emerged as promising treatments for AML patients with *C-KIT* mutations. Patients harboring different *C-KIT* mutations may show variable responses to TKIs [7]. *C-KIT* mutation is often detected as a single mutation in one patient. Indeed, higher frequencies of double mutations in *C-KIT* gene have been reported in patients with AML [5], gastrointestinal stromal tumors [8,9], and melanoma [10]. Aberrant activation of receptor tyrosine kinases is a common feature of many cancer cells. More frequent mutations have a higher activating effect [11]. However, the clonal evolution, clinical characteristics, and prognostic significance of double *C-KIT* exon 17 mutations in CBF-AML remain unclear. In this study, we detected six cases harboring double *C-KIT* exon 17 mutations by ddPCR and sequencing in 75 patients with CBF-AML. Furthermore, the dynamic evolution, clone composition, and clinical significance of double *C-KIT* mutations were analyzed.

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2. Materials and methods

2.1. Patients and treatment

From January 2012 to October 2017, a total of 75 patients were diagnosed with CBF-AML at the Second Hospital of Shanxi Medical University. Patients were diagnosed according to the morphological, immunological, cytogenetic, molecular criteria. For the diagnosis of CBF-AML, t(8;21) or inv(16)/t(16;16) was detected by karyotyping, while the detection of *AML1-ETO* or *CBFβ-MYH11* fusion transcripts was conducted by reverse transcription polymerase chain reaction (RT-PCR). Among the samples, 60 cases were positive for *AML-ETO* and 15 cases for *CBFβ-MYH11*. All patients were treated with standard chemotherapy based on anthracycline drugs, cytarabine, and/or tyrosine kinase inhibitors. The study protocol was approved by the Research Ethics Board of the same hospital, and in accordance with the ethical principles stated in the Declaration of Helsinki. Written informed consent was obtained from all patients.

2.2. DNA and RNA extraction

Genomic DNA and total RNA were extracted from bone marrow cells by using E.Z.N.A. DNA/RNA Isolation Kit (Omega Bio-tek). The purity and concentration of DNA and RNA samples were measured using a spectrophotometer.

2.3. Screening of mutations in C-KIT gene and other genes related to the prognosis of CBF-AML

Exon 17 of *C-KIT* was amplified using the following primers: forward 5′-ATGTGAACATCATTCAAGGCGTACT-3′ and reverse 5′- GCTA AAATGTGTGATATCCCTAGACAG-3′. All PCR reactions were performed in a final volume of 25 μL reaction mixture containing 2.5 μL of 10 × PCR buffer with $(NH_4)_2SO_4$, 3 μL of 1.5 mmol/L $MgCl_2$, 1.5 U of Taq polymerase (Fermentas), 2.5 μL of 10 mmol/L dNTPs Mix (Promega), 20 mmol/L of each primer, 2 μL of template DNA and ddH_2O. The PCR cycles were as follows: 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, with a final extension step at 72 °C for 10 min. The PCR products were run on 2% agarose gels and were then subjected to direct sequencing.

Several genes such as *FLT3*, *NPM1*, *TP53* and *ASXL1* have been reported to be closely related with prognosis of AML. Thus, the hotspot mutation of each gene was screened by using DNA or RNA as the amplification template. The sequences of all primers are listed in Supplementary Table A1. The methods for detection of point mutations were similar to those described under *C-KIT* gene.

2.4. Cloning and sequencing

The PCR products were sequenced by Beijing Augct biotechnology Co., Ltd. Gene cloning and sequencing was performed on an as-needed basis. Sequence analysis was carried out by using Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.5. The analysis of C-KIT gene mutations by ddPCR

To determine the composition and evolutionary changes of double mutation in *C-KIT* gene, ddPCR was performed in all patients with CBF-AML. The ddPCR reaction mixtures of 20 μL volume comprising 100 ng DNA, 1 × ddPCR supermix, 0.4 μM of each primer, and 0.2 μM of each probe (Table A1 in the Supporting Information) were prepared in water-in-oil emulsions using droplet generator cartridges (Bio-Rad). The generated droplets were then transferred to a 96-well plate and amplified by PCR. The amplification conditions were as follows: 95 °C for 5 min, 95 °C for 30 s, 62 °C for 30 s and 72 °C 45 s (40 cycles), and 72 °C for 10 min. Subsequently, the PCR products were detected on the QX100 droplet reader (Bio-Rad). Samples with a droplet number of more than 3 were considered positive by QuantaSoft analysis software.

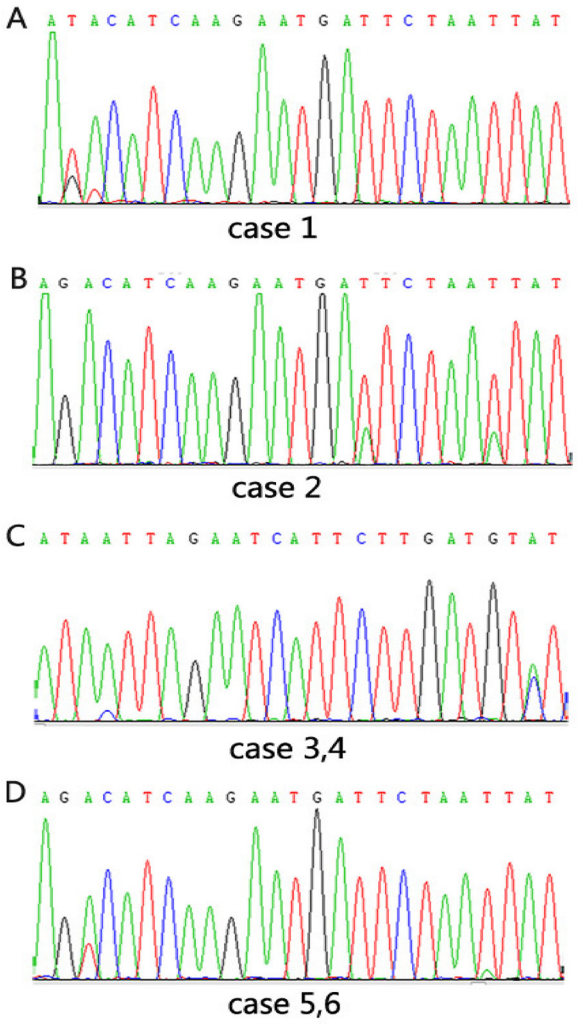

Fig. 1. Sequencing results of CBF-AML patients with double *C-KIT* mutations in exon 17.

3. Results

3.1. Frequencies of C-KIT gene mutations in CBF-AML patients

C-KIT mutations were detected in 32 (42.7%) of 75 cases, of which 43.3% (26/60) involved t(8;21) and 40% (6/15) involved inv(16). Among these 32 patients, a total of 38 point mutations were identified, including codons 816 (D816 V, $n = 16$; D816H, $n = 4$; D816Y, $n = 7$; D816F, $n = 1$), N822 K ($n = 9$) and D820E ($n = 1$). In addition, the sequencing results revealed that six (18.8%) cases possessed double point mutations in exon 17. One case carried double mutations in the same codon of D816 (Fig. 1-A), one case in codons D820 and N822 (Fig. 1-B), and the other four cases (one of them was detected by ddPCR) in codons D816 and N822 (Fig. 1-C,D).

3.2. Cloning and sequencing results of CBF-AML patients with double C-KIT mutations

The PCR fragment of exon 17 was subcloned into a T-vector followed by sequencing, in order to assess whether the double mutations are located on the same allele and to identify their genetic composition. The results showed that three clones possessed wild type, three clones