METHODOLOGY

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Development of a highly sensitive method for detection of FLT3D835Y

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Abstract

Background: Acute myeloid leukemia (AML) is a malignant hematological neoplasm of myeloid progenitor cells. Mutations of FLT3 in its tyrosine kinase domain (FLT3-TKD) are found in ~8% of patients with AML, with D835Y as the most common substitution. This mutation activates survival signals that drives the disease and is resistant to the first generation FLT3 inhibitors. Development of a highly sensitive method to detect FLT3D835Y is important to direct therapeutic options, predict prognosis, and monitor minimal residual disease in patients with AML.

Methods and results: In the present study, we developed a highly sensitive FLT3D835Y detection method by using the restriction fragment nested allele-specific PCR technique. The method consists of three steps: 1) initial amplification of DNA samples with PCR primers surrounding the FLT3D835Y mutation site, 2) digestion of the PCR products with restriction enzyme EcoRV that only cleaves the wild type allele, and 3) detection of FLT3D835Y by allele-specific PCR with nested primers. We were able to detect FLT3D835Y with a sensitivity of 0.001% by using purified plasmid DNAs and blood cell DNAs containing known proportions of FLT3D835Y. We analyzed blood cell DNA samples from 64 patients with AML and found six FLT3D835Y-positive cases, two of which could not be detected by conventional DNA sequencing methods. Importantly, the method was able to detect FLT3D835Y in a sample collected from a relapsed patient while the patient was in complete remission with negative MRD determined by flow cytometry. Therefore, our RFN-AS-PCR detected MRD after treatment that was missed by flow cytometry and Sanger DNA sequencing, by conventional methods.

Conclusions: We have developed a simple and highly sensitive method that will allow for detection of FLT3D835Y at a very low level. This method may have major clinical implications for treatment of AML.

Keywords: Tyrosine kinase, Acute myeloid leukemia, FLT3-TKD, Detection of mutations

Background

Acute myeloid leukemia (AML) is the most frequent form of leukemia in adults, representing approximately one-third of all leukemia cases in the United States [1–3]. It is featured by the presence of myeloblasts in marrow and/or peripheral blood that impair normal hematopoiesis [4–6]. Although significant progress has been made in treatment of patients with a favorable prognosis [7–10], the overall survival of the disease remains poor [11].

To date, several molecular lesions have been identified in patients with AML [12–14]. Among them, FMS-like tyrosine kinase 3 (FLT3) is one of the most frequently mutated genes, found in around one-third of patients [9, 15, 16]. FLT3 is a receptor tyrosine kinase that has a crucial role in the development of hematopoietic progenitor cells. Gain-of-function mutations of FLT3 include internal

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tandem duplication (FLT3-ITD) and point mutations in the kinase domain (FLT3-TKD), each of which could lead to over-activated signals for proliferation and survival of leukemia cells [2, 17]. Studies have validated that both FLT3-ITD and FLT3D835Y are driver mutations in AML that cause myeloid neoplasms in murine models [18, 19], FLT3-ITD occurs in about 25% of AML patients and represents an unfavorable prognosis unless nucleophosmin 1 (NPM1) mutations are present [20, 21]. FLT3-TKD mainly includes the FLT3D835Y substitution and is found in ~7% of patients with AML [20, 21], FLT3 mutations could serve as important diagnostic and prognostic markers for AML. Elimination of cells carrying mutant FLT3 could be used to evaluate the clinical response and minimal residual disease (MRD) of patients during treatment. To date, several methods to detect FLT3-ITD have been developed [22], but convenient and sensitive methods to detect FLT3-TKD are still needed.

Current methods for FLT3-TKD genotyping include Sanger sequencing, restriction enzyme fragmentation, next generation sequencing, real-time polymerase chain reaction (real-time PCR), and denaturing high performance liquid chromatography [19, 23–26]. These methods have reported sensitivities ranging from 0.3 to 5%, and each has its own advantages and pitfalls. Some are not sensitive enough to detect MRD while others are sensitive but give nonspecific false positives. Also, some of these methods are labor-intensive and time-consuming, and they may require specialized or costly equipment and reagents [19, 25]. A more reliable and more sensitive method is still needed for the detection of mutant FLT3. In the present study, we developed a simple and highly sensitive method to detect FLT3D835Y based on the restriction fragment nested allele-specific PCR (RFN-AS-PCR) technique.

Methods

Sample collection and DNA extraction

Blood samples were collected from the clinical laboratory of the Seventh Affiliated Hospital of Sun Yat-sen University under an approved Institutional Review Board protocol. Genomic DNAs were purified by using the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0 (TaKaRa, Shiga, Japan). For each PCR reaction described below, around 1 μg total DNA was used.

FLT3 and FLT3D835Y plasmid DNA standards

An 856-bp DNA fragment covering the D835 region of FLT3 was amplified from genomic DNA with primers F1_5 (5′- AGAAGGCCGCCAAGAAC) and F1_3 (5′- CACCCAGCCCAATTCCTAC) and then cloned into the pGem-T vector (Promega, WI, USA). Plasmid DNAs were purified from E. coli cells by using the TaKaRa MiniBEST Plasmid Purification Kit Ver.4.0 (TaKaRa, Shiga, Japan). The plasmid containing FLT3D835Y was generated by PCR using Q5® Site-Directed Mutagenesis Kit (NEB, MA, USA) with primers 5′- ATTGGCTCGATATCATGAGTG and 5′- CCAAAATGCACATATCTTCAC. Purified FLT3 and FLT3D835Y plasmid DNAs were sequencing verified and mixed at different proportions and diluted to 20 μg/ml and 1 μl of the DNA sample mixtures were used for PCR analysis described below. DNA concentrations were determined by measuring absorbance at 260 nm.

Initial PCR, EcoRV restriction enzyme digestion, and nested AS-PCR

The method of RFN-AS-PCR was described previously [27]. A schematic illustration is provided in Fig. 1a. In brief, isolated plasmid or blood cell DNAs were used as a template for initial PCR with primers F1_5 and F1_3. The PCR was run with Taq DNA polymerase for 35 cycles with each cycle consisting of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The PCR products were then digested in a 10 μl reaction mixture containing 1 μl PCR products and 0.4 unit of EcoRV (NEB) at 37 °C for 1 h. The digested PCR products were further subjected to AS-PCR with nested primers F1_5n (5′- GCACCTCGGATAATCAC) and F1_3n (5′- AGCCTAGAGCATATGTGATG) and allele specific primers F1_mut (5′- ATATGTGACTTGGAATGTCGCTAT) and F1_wt (5′- CATAGTGGAATCTACATGATC). The PCR was run for 35 cycles with each cycle consisting of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The final PCR products were resolved on 1% agarose and visualized with SYBR green (Thermo Fisher Scientific, USA) staining. To ensure no cross-contamination occurred, control experiments with water replacing DNA samples were routinely performed and filter tips were used throughout. For sequencing verification, PCR products were gel-purified with TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (TaKaRa, Shiga, Japan) and then analyzed by using an ABI3730 capillary sequencer (Geneviz, Suzhou, China). Each of the above experiments was repeated at least three times with consistent results.

Detection of MRD with flow cytometry

Bone marrow samples from patient No.11 during the remission and relapse stages were analyzed by KingMed Diagnostics (Guangzhou, China).

Results

Development of restriction fragment nested allele-specific PCR (RFN-AS-PCR) for detection of FLT3D835Y

The FLT3D835Y mutation corresponds to c.2503G > T in DNA and disrupts an EcoRV restriction site (GATATC to TATATC). This provides a convenient way for detection of FLT3D835Y using the so-called restriction fragment length polymorphism technique that has a sensitivity of around 1%. On the other hand, allele-specific PCR is another commonly used method for detection of DNA point mutations
with a sensitivity of around 1%. To enhance the sensitivity and specificity, we developed the method by combining restriction fragment length polymorphism with allele-specific PCR, and designated the method restriction fragment nested allele-specific PCR (RFN-AS-PCR). A schematic diagram of the procedure is illustrated in Fig. 1a. We first employed purified plasmid FLT3 and FLT3D835Y DNAs to determine the sensitivity of the method. The standards were created using a mixture of the two purified plasmid DNAs. Our data demonstrated that nested AS-PCR without EcoRV digestion had a sensitivity of 1% (Fig. 1b). With the EcoRV digestion step introduced, the RFN-AS-PCR technique increased the detection limit to 0.001% (Fig. 1c).
Validation of the RFN-AS-PCR method using AML patient samples

We further validated the method by using a FLT3D835Y-positive AML patient blood DNA sample identified by our RFN-AS-PCR method (Fig. 2a). Sanger sequencing revealed that this particular sample (AML11) had a mutation rate of around 50% (Fig. 2b). We then mixed in various proportions of this FLT3D835Y-positive DNA sample and a FLT3-wild type blood DNA sample to create standards of genomic DNAs. A total of 1 μg of the DNA mixtures was used for the initial PCR, followed by nested AS-PCR.

**Fig. 2** Validation of the RFN-AS-PCR method by using mixtures of DNA samples from FLT3D835Y mutant and wild type AML blood samples. a Screening of 13 AML blood samples by using RFN-AS-PCR identified AML11 as a FLT3D835Y-positive case. b Verification of FLT3D835Y positivity in AML11 by Sanger sequencing. c and d Blood cell DNAs from AML11 and a patient with wild type FLT3 were mixed in the indicated proportions. AS-PCR was performed without EcoRV digestion (panel c) or with EcoRV digestion (panel d). Note that the EcoRV digestion increased the detection sensitivity from 1 to 0.001%
PCR with or without prior EcoRV digestion. Consistent with results obtained using standard plasmid DNA samples described above, nested AS-PCR alone was able to detect mixtures containing 1% of FLT3D835Y (Fig. 2c), while with the EcoRV digestion step, RFN-AS-PCR increased the detection sensitivity to 0.001% (Fig. 2d).

Screening of AML samples for FLT3D835Y by RFN-AS-PCR

We then applied the RFN-AS-PCR method to screen AML patients for FLT3D835Y positivity. Out of 64 samples collected from a cohort of 64 AML patients (see Figs. 2a, 3a, and 4a), we found 6 FLT3D835Y-positive cases, corresponding to a 9.4% rate, which is higher than the reported rate of all FLT3-TKD mutations in patients

Fig. 3 Screening of AML samples for identification of FLT3D835Y by using RFN-AS-PCR. a Detection of FLT3D835Y in 40 patients with AML by RFN-AS-PCR. b Failure of Sanger sequencing to detect FLT3D835Y in AML24 and AML45
with AML of 3.8 to 7.7% [28–30]. Figure 3a shows data from 36 of these samples. The relative low sample size may contribute to this high mutation rate. However, we found two of the positive samples had a low level of mutation that was not detectable with Sanger sequencing (Fig. 3b). This partly explains the high percentage of samples with the FLT3D835Y mutation rate observed in this study. It should be pointed out that this a low level of FLT3D835Y may not be the primary cause of the AML phenotypes for patients when the samples were collected. It probably co-exists with other mutations (e.g., FLT3-ITD) and may be responsible for relapse and progression of the disease to some degree. This potentially explains why some FLT3-ITD-positive patients treated with the first-generation FLT3 inhibitors often relapse with appearance of FLT3D835Y.

**Detection of MRD by RFN-AS-PCR in an FLT3D835Y-positive AML patient in complete remission**

We further applied the RFN-AS-PCR method to analyze DNA samples collected from a 69-year-old AML patient before and after treatment. The patient was determined to have the FLT3D835Y mutation by Sanger DNA sequencing of blood DNA samples (Fig. 4). After treatment with decitabine plus homoharringtonine, cytarabine, and G-CSF, complete remission was achieved with negative MRD determined by flow cytometry analysis and DNA sequencing. Unfortunately, the disease relapsed 2 weeks later with 18.4% myeloblasts in bone marrow cells and positive Sanger DNA sequencing results. However, when blood DNA samples were analyzed by using the RFN-AS-PCR method, FLT3D835Y was found in all samples including the one at remission despite a relatively weak signal. Our RFN-AS-PCR detected MRD after treatment that was missed by flow cytometry and Sanger DNA sequencing. Therefore, the RFN-AS-PCR method should be a more reliable method to detect MRD for FLT3D835Y-positive AML patients.

**Discussion**

In the present study, we developed a highly sensitive RFN-AS-PCR method to detect FLT3D835Y by combining restriction enzyme digestion and AS-PCR. The method is simple, quick, and inexpensive, and it does not require specialized equipment and reagents. Our data show that the method has a sensitivity of up to 0.001% and is able to detect FLT3D835Y in samples that are not detectable with other conventional methods. With the RFN-AS-PCR method, we found that 9.4% (6/64) of AML were FLT3D835Y-positive, which is higher than the reported
3.8 to 7.7% rate for all combined FLT3-TKD mutations in AML [28]. The data suggest that FLT3D835Y is more common than previously thought.

Both FLT3-TKD and FLT3-ITD are gain-of-function mutations of FLT3. However, their activation mechanisms differ and do so their responses to FLT3 inhibitors [17]. In fact, several FLT3 inhibitors including sorafenib and quizartinib potently inhibit FLT3-ITD but are not effective toward FLT3-TKD [31]. Consequently, FLT3-TKD-positive AML patients are resistant to treatment with sorafenib and quizartinib. Furthermore, many FLT3-ITD-positive AML patients relapse with the appearance of FLT3-TKD mutations after initial response to FLT3 inhibitor treatment. A plausible explanation for this is that FLT3-TKD mutations co-exist with FLT3-ITD at a very low level and become prevalent after FLT3-ITD-positive leukemic cells are killed. The two AML cases with very low level of FLT3D835Y identified in this study may belong to this scenario. Therefore, the presence of FLT3D835Y should be used to guide treatment plans for patients with AML.

Detection of MRD has major implications for recommending therapies and predicting outcomes in the treatment of AML [32]. Current methods for assessing MRD include multicolor flow-cytometry (MFC), real-time PCR, and next-generation sequencing (NGS) [33]. Although NGS is capable of detecting multiple molecular lesions with the sensitivity level of around 1%, it is considered labor-intensive with a longer turnaround time compared with other methods [34]. The detection of residual leukemic cells by MFC relies on the detection of antigens that are absent or rare in normal bone marrow cells. It is fast and cost-effective, but immunophenotypes of leukemic cells could change during the treatment, which potentially contributes to inaccuracy of the method [35]. Currently, the occurrence of relapse in AML patients with negative MRD ranges roughly between 20 and 25%, reflecting a major problem with the sensitivity and accuracy of currently available MRD detection techniques [33]. Our RFN-AS-PCR for FLT3D835Y has the sensitivity of 0.001% that by far outperforms the existing methods. In this regard, our study provides a powerful tool to detect FLT3D835Y to support diagnosis, therapeutics, and MRD-monitoring for patients with AML.

**Conclusions**

We have developed a simple and highly sensitive method that will allow for detection of FLT3D835Y at a very low level. This method may have major clinical implications for diagnosis and treatment of AML.

**Abbreviations**

AML: acute myeloid leukemia; FLT3: FMS-like tyrosine kinase 3; FLT3-ITD: FLT3 internal tandem duplication; FLT3-TKD: FLT3 tyrosine kinase domain mutations; NPM1: nucleophosmin 1; PCR: polymerase chain reaction; MRD: minimal residual disease; RFN-AS-PCR: restriction fragment nested allele-specific PCR; MFC: multicolor flow-cytometry; NGS: next-generation sequencing.

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Not applicable.

**Authors’ contributions**

Y.G., Y.C., and Z.J.Z conceived the project. Y.G., H.S., D.Z., Y.Z., and M.Y. performed the experiments. H.S., M.S., S.X., X.F., B.L., T.B., X.X., S.X., and X.X. collected clinical samples. Y.G., H.S., D.Z., X.X., Y.C., and Z.J.Z analyzed data. Y.G., Y.C. and Z.J.Z wrote the manuscript. The author(s) read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analysed during this study are included in this published article and its supplementary information files.

**Ethics approval and consent to participate**

Blood samples were collected from clinical laboratory of the Seventh Affiliated Hospital of Sun Yat-sen University under an approval from Medical Ethics Committee of the Seventh Affiliated Hospital, Sun Yat-sen University (2018SYSUSH-013). Informed consent was obtained from all participates.

**Consent for publication**

Informed consent form for publication was obtained from all participants.

**Competing interests**

The authors declare that they have no competing interests.

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