Simplified capillary electrophoresis detection of the Flt-3 internal tandem duplications and D835 point mutations in acute myeloid leukemia

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Introduction

Flt-3 mutations, which include the internal tandem duplications (ITD)[1] and missense mutations at the D835 (aspartic acid) codon,2 are arguably the most common somatic mutations in acute myeloid leukemia (AML).[3-4] As relapse remains the principal cause of treatment failure for the majority of AML patients, identification of patients with a high risk of relapse would be useful for the introduction of alternative forms of therapy in this group of patients. As the presence of Flt-3/ITD is the most consistent factor in predicting relapse in AML, it is thus a valuable prognostic marker for the disease.[5-7] Correlation of the D835 point mutation with poor event-free survival[2] suggests that it may also contribute to the constitutive activation of the Flt-3 receptor tyrosine kinase, resulting in poorer prognosis.

Several independent studies have utilized polymerase chain reaction (PCR) amplification and agarose gel electrophoresis for the detection of Flt-3 gene mutations.[1-8] Capillary electrophoresis (CE) was used by one group to confirm the results of ITD analysis on agarose gel[6]. However, the use of CE as a first-line detection procedure for both ITD and point mutations has not been previously reported. We report our experience using a simplified CE-based assay for rapid detection of Flt-3/ITD and D835 mutations in 33 AML patients seen at the National University Hospital, Singapore.

Materials and Methods

Genomic DNA isolation was carried out using the PUREGENE™ DNA Isolation kit (Gentra, Minneapolis, USA). To detect ITD, we amplified exons 11 and 12 of the Flt-3 gene (including the intervening intronic sequence) using primer pairs Int10.39 (5' TCT GCA GAA CTG CCT ATT CCT 3') and 5' 5-carboxyfluorescein-labeled 12R-FAM (5' CTT TCA GCA TTT TGA CGG CAA 3'). To detect D835 mutations, we amplified the entire exon 17 using primers 17F-HEX (5' CCG CCA GGA ACG TGC TTG 3') and 17R-FAM (5' GCA GCC TCA CAT TGC CCC 3') and digested the amplification products for 1 hr at 37°C with 10 U of EcoRV (New England Biolabs). Next 0.5 mL of the PCR-amplified products were added to 12 μL of deionised formamide and 0.5 mL of GeneScan ROX internal size standard (500™ ROX for ITD; 350™ ROX for D835). Following denaturation (2 min at 95°C) and cooling on ice, the products were size-separated using the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems), and the separation profiles analyzed with the GeneScan v3.7 Analysis Software™.

Results and Discussion

We detected 5 ITD (15.2%) from the 33 patients studied, a mutation rate very similar to those found in other populations.[1,4,5,8] Sequence analysis of the PCR products from all 5 samples confirmed that various partial sequences within exon 11 were tandemly duplicated. Figure 1a shows examples of CE profiles. Figure 1b shows the agarose gel resolution of two wild-type samples (L1, L7) and ITD-containing (L2-L6) samples. The wild-type samples yield a single product at approximately 377 bp whereas samples with ITD yield an extra band of larger size. When compared to the CE results (Figure 1a), the actual sizes of the tandemly duplicated products are more difficult to determine accurately from an agarose gel. This is clearly shown by samples L2 and L4 (Figure 1b), where differences of 18 bp and 21 bp, respectively, between the germline products and the tandemly duplicated products could have been misinterpreted as a single band if the separation time was shortened. This clearly demonstrated the superior resolution of CE.

We found one patient with a D835 mutation in our study population (3%). This sample showed a partial digestion, producing a wild-type fragment at 114 bp and two digestion products of 68 bp and 46 bp sizes, (Figure 2). Sequence analysis revealed a GAT to TAT base substitution at codon D835, resulting in a D835Y (aspartic acid to tyrosine) change. In concordance with the incomplete digestion profile observed by CE analysis (Figure 2), the sequence data showed G and T overlapping at the first base of codon D835, confirming the mutation in only one allele.

Our results demonstrated that CE permits a more precise determination of the product size without having the problems of band shift artefacts and gel-to-gel variation, often associated with agarose or polyacrylamide gel electrophoresis. The higher resolution of CE also allows separation and identification of PCR products differing in length by only 1 bp. These performance-linked improvements are particularly helpful in the identification of tandemly duplicated products, which can be very similar in size to the wild-type products.

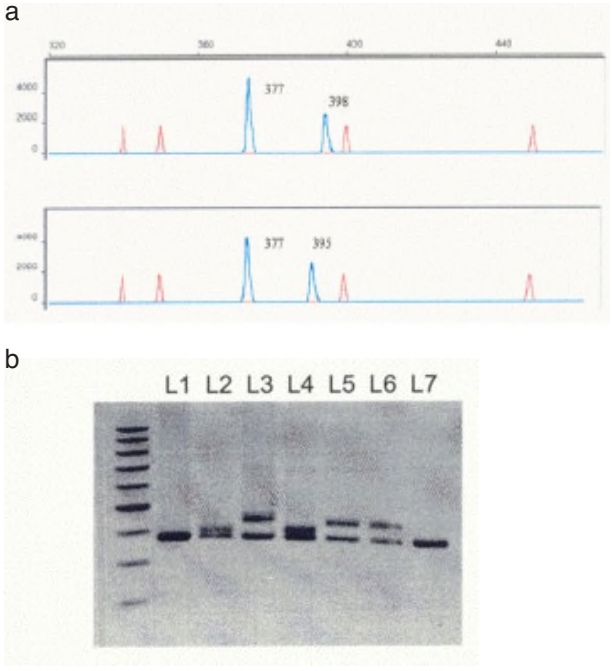

Figure 1. (a) CE profiles showing patient samples with Flt-3/ITD mutations. Two distinctive peaks are observed in all Flt-3/ITD positive samples representing germline products at 377 bp, and tandemly duplicated products at a point greater than 377 bp. (i) the same Flt-3/ITD positive sample as sample L2, Figure 1(b); (ii) the same Flt-3/ITD positive sample as sample L4, Figure 1(b); The CE profiles provide a much clearer and more accurate size evaluation compared to that estimated from bands detected by agarose gel electrophoresis. (b) A 2% agarose gel photo showing wild-type samples and Flt-3/ITD positive samples. L1 & L7: wild-type patient samples which yield a product of approximately 377 bp in size. L2 - L6: patient samples harboring a Flt-3/ITD mutation. These samples yield an extra product that is bigger than the germline product at 377 bp.

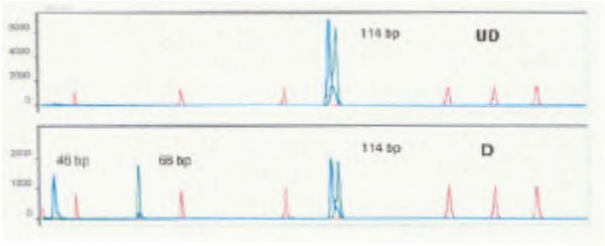

Figure 2. Detection of D835 point mutation by CE. An electropherogram showing the undigested (UD) and digested (D) profiles of a D835 positive sample. The D835Y point mutation was heterozygous, producing a partially digested product, leaving the germline product (114 bp) as well as the digested products (68 bp & 46 bp).

Our results indicate that the proposed CE-based test can be reliably used to detect both types of Flt-3 gene mutations and is suitable for clinical purposes.

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