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Establishment of a quenching probe method for detection of *NPM1* mutations in acute myeloid leukemia cells

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Abstract. Nucleophosmin (*NPM1*) mutations, generally consisting of a four base-pair insertion, are present in ~60% of all cytogenetically normal acute myeloid leukemia (AML) cases. The mutation is clinically significant as an important prognostic factor. Direct sequencing is the current standard method of mutation detection, however, it is quite costly and time consuming. The present study aimed to establish a highly sensitive quenching probe (QP) method to detect *NPM1* mutations efficiently. Melting curve analysis was performed using a QP, following polymerase chain reaction for amplification of the involved region of the gene. The curve derived from the fluorescent intensity with respect to the temperature of OCI/AML3, a heterozygous *NPM1* mutant AML cell line, was W-shaped with melting peaks at 61˚C and 68˚C. That of M-07e, the homozygous wild type cell line, was V-shaped with a melting peak at 68˚C. Thus, the curve derived from the mutant allele was easily discriminated from that of the wild-type allele. The mutant allele was detected in concentrations as low as 3% as determined by a subsequent sensitivity study. With a short testing time and a high sensitivity, this assay was applicable for *NPM1*-mutated AML patient samples and is appropriate for screening *NPM1* mutations. It does require further examination as to whether it would be useful as a detection method for other mutant alleles since *NPM1* mutations may consist of 61 known types of mutant sequences. To the best of our knowledge, this is the first report describing the QP method for the detection of *NPM1* mutations.

Introduction

Mutations found in the nucleophosmin gene (*NPM1*) are novel molecular abnormalities identified in acute myeloid leukemia (AML). *NPM1* mutations are present in ~60% of cytogenetically normal AML cases, and they are an important prognostic factor (1,2). Therefore, AML with mutated *NPM1* has been defined as a provisional entity in the 4th edition of the World Health Organization classification (3). The mutations typically appear as a 4 base pair (bp) insertion at position 956 through 971 in exon 12, resulting in frame-shift mutations (1). To date, 61 known types of mutations have been reported (2,4-7). The most common *NPM1* mutation is an insertion of TCTG at position 956-959, referred to as type A (see Table I), and it is detected in ~80% of *NPM1* mutation cases (1,8,9).

Direct sequencing is the current standard method for detecting *NPM1* mutations. Unfortunately, this method is costly and time consuming. In addition, the sensitivity of this method is low, with the lower limit of detection beginning at ~20% (10). Therefore, polymerase chain reaction (PCR) methods such as electrophoresis (11), melting curve analysis (12), high-resolution melting (HRM) analysis which detects the different melting temperatures of the PCR products (13), locked nucleic acid clamp-mediated PCR (10,14), and capillary electrophoresis (15,16) have been investigated as potentially more sensitive methods for detection of this 4 bp mutation.

Recently, the quenching probe (QP) method has been developed as a novel technique for mutation detection (17,18). A QP is an oligonucleotide with a fluorescent dye-modified cytosine at the 5' or 3' end. After PCR amplification of the sequences including the mutation site, a melting curve analysis using QPs is performed. At low temperature, QPs hybridize with PCR products, and their fluorescence is quenched by an electron transfer to adjacent guanine bases in the PCR products. With an increase in temperature, the QPs dissociate from the products, causing an increased fluorescent signal emission (19). Because QPs dissociate from unmatched products at lower temperatures than perfectly matched products, it is possible to detect mutant alleles (20). In the present study, the sensitivity and effectiveness of a newly established QP method was examined.

Materials and methods

Cells and DNA extraction. Two leukemia cell lines were used: an AML cell line, OCI/AML3, with a heterozygous type A *NPM1* mutation (9) and a megakaryoblastic leukemia cell

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line, M-07e, with homozygous wild-type *NPM1*. DNA was extracted from the cells by the SepaGene agglutination partition method (EIDIA, Tokyo, Japan). To examine the sensitivity of the detection method, mixed samples of OCI/AML3 DNA and M-07e DNA in various ratios were used. Blood samples were obtained from 5 AML patients prior to treatment, with their informed consent. The DNA extracted from these samples was also examined. The study was approved by the Ethics Review Board in Tokyo Medical and Dental University (Tokyo, Japan).

QP method. The QP method from a previously reported protocol (18) was performed using a LightCycler Nano™ (Roche Diagnostics, Mannheim, Germany). The sequences of PCR primers and QPs are presented in Table I. A total of 3 QPs with different sequences were designed to compare efficiency. The PCR cycling conditions were as follows: A 95˚C hold (10 min); 10 cycles at 95˚C (10 sec), 65-55˚C ramp (10 sec, 1˚C/step), 72˚C (30 sec); followed by 45 cycles of 95˚C (10 sec), 55˚C (10 sec), and 72˚C (30 sec). The reaction volume consisted of 19.5 μl with 18 ng DNA sample, 1.5 mM $MgCl_2$, 0.2 μM primers, 0.2 μM QP, and 4 μl reaction mix of LC 480 Genotyping Master (Roche Diagnostics). Upon completion of the PCR cycles, the temperature was maintained at 95˚C for 1 min, followed by 45˚C for 1 min, and then gradually increased to 95˚C at a rate of 0.2˚C/sec, during which the fluorescent signal was continually acquired. The curves derived from the fluorescent intensity with respect to temperature, -dFluorescence/dTemperature (-dF/dT), were obtained using LightCycler Nano™ software version 1.0 (Roche Diagnostics). Each assay was performed in duplicate to verify the reproducibility of the method.

Direct sequencing. For confirmation of sequences, the PCR products were treated with Amicon Ultra Centrifugal Filter Units (Sigma-Aldrich, St. Louis, MO, USA) to remove the primers and dNTPs, and then sequenced using a 3130x1 Genetic Analyzer and BigDye terminator Reaction kit v3.1 (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's protocol.

Results

Discrimination between wild-type and mutant alleles. The curves for -dF/dT as determined by QP analysis using the three variations of QPs (QP 1, QP 2, and QP 3) are shown in Fig. 1. DNA from M-07e cells possessing the homozygous wild-type alleles presented with single concave-up curves, with the lowest point at about 65-68˚C. DNA from OCI/AML3 cells possessing the wild-type allele and the type A mutant allele presented two concave-up curves with lowest points at 65-68˚C and 55-61˚C, respectively. In comparing the melting profiles of the three variations of QPs investigated, QP 2 was the most discriminative probe; the wild-type allele and type A mutant allele presented curves with the lowest points at 68˚C and 61˚C, respectively. Therefore, further QP analysis was performed using QP 2.

Assay sensitivity. In order to evaluate the sensitivity of the QP assay, the OCI/AML3 DNA and M-07e DNA were mixed at various ratios. Fig. 2 shows the representative results of the lower detection limit obtained using these mixed samples containing OCI/AML3 DNA and M-07e DNA. Recognizable curves with the 61˚C melt point were obtained from samples containing as low as 6.25% OCI/AML3 DNA, or 3.125% of mutant allele.

Table I. Primers, probes, and sequences of the mutated region.

Variable	Sequence
PCR primers	
Forward	5'-tgatgtctatgaagtgttgtggttcc-3'
Reverse	5'-ctctgcattataaaaaggacagccag-3'
Quenching probes	
1	acttcctccactgccagagatc-(BODIPY FL)
2	(BODIPY FL)-cctccactgccagagatcttgaa-P
3	(BODIPY FL)-ctattcaagatctctggcagt-P
Sequences	
Wild-type	caggctattcaagatctctggcagtggagg
Type A mutant	caggctattcaagatctctgtctggcagtggagg

BODIPY FL is a green fluorescent dye. P, phosphorylation.

Detection of NPM1 mutations in patients' samples. Out of the five patient samples examined, three samples presented the W-shaped curves with lowest points at 61˚C and 68˚C, indicating that these samples contained both the mutant alleles and the wild-type alleles. Fig. 3 shows the curves of sample 1 and sample 2, both containing wild-type and mutant alleles. The curve of sample 3 was similar to that of sample 1 (data not shown). Direct sequencing further confirmed that these three samples have a type A mutant allele (data not shown). The remaining two samples presented V-shaped curves with lowest points at 68˚C, indicating that these samples contained wild-type allele only.

Discussion

In the present study, a QP method was established to detect *NPM1* mutations easily with high sensitivity. To the best of our knowledge, this is the first report to use the QP method to detect *NPM1* mutations. The curve derived from the mutant allele was easily discriminated from that of the wild-type allele. Thus far, the authors of the present study performed screening for *NPM1* mutations by gel electrophoresis of PCR products to detect a band 4-bp longer than the wild-type band; however, it was not clearly discriminative (data not shown). HRM analysis of the PCR products to detect the difference of the melting curves (13) was additionally performed by the present authors, but again, it was not clearly discriminative (data not shown). Compared with these two analyses, the QP method was superior in its specificity.

In terms of efficiency, the QP method took just 2 hours to perform. The sensitivity was quite high as the lower limit of detection was as low as a 3% concentration of mutant allele. In clinical settings, *NPM1* assays are predominantly used for diagnosis at presentation, rather than for detection of minimal

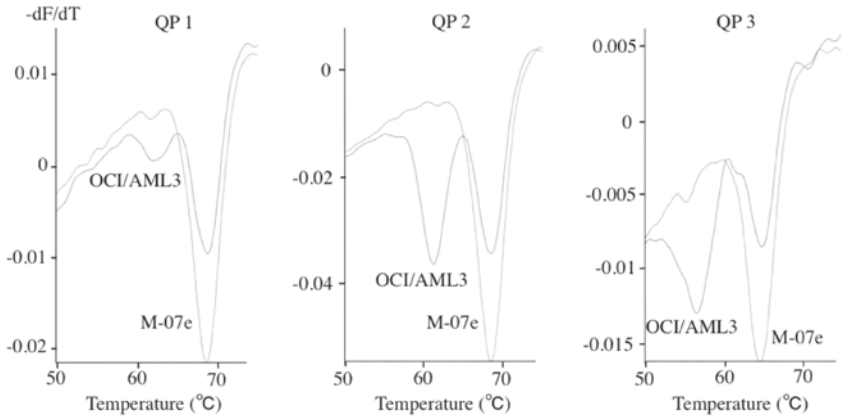

Figure 1. Comparison of derivative melting curves using three kinds of quenching probes (QP). -dF/dT indicates -d (Fluorescence)/d (Temperature).

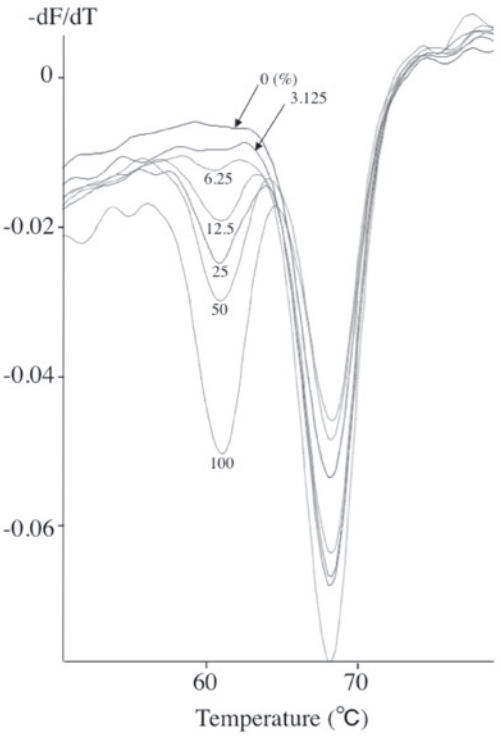

Figure 2. Sensitivity of quenching probes analysis for the detection of *NPM1* mutations. Numbers shown indicate the percentage of OCI/AML3 DNA.

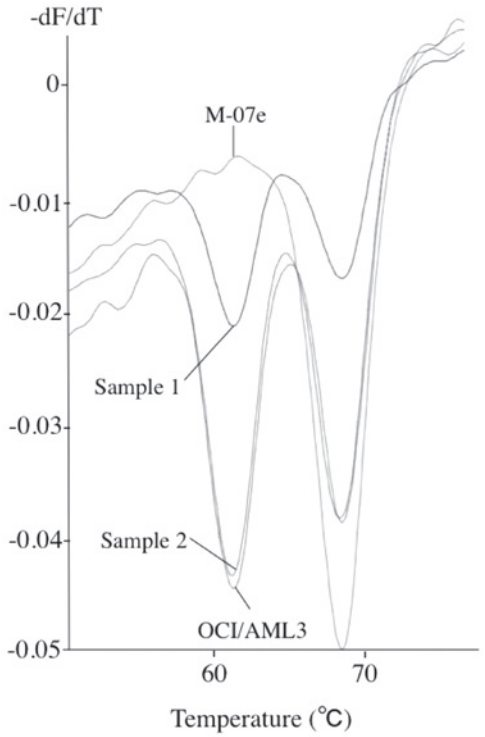

Figure 3. Derived melting curves from quenching probes analysis for the detection of *NPM1* mutations in acute myeloid leukemia patient samples and cell lines.

residual diseases. The mutant alleles are typically present in at least 10% of the cells in AML blood or bone marrow samples, even in heterozygous mutant cases. Therefore, higher sensitivity is not necessarily needed for diagnosis. Moreover, by comparing the depth of the two curves as shown in Fig. 2, the approximate ratio between mutant allele and wild-type allele can be estimated.

The QP assay also demonstrated its applicability for clinical use. In the AML patient samples, the mutant allele was detected in three samples, which were all confirmed to be type A mutant cases. Non-type A mutant AML samples were not encountered in this study as the number of available AML samples were few. To date, at least 61 types of mutant sequences of *NPM1* gene have been reported (2,4-7). The QP 2 sequence used in this assay covers the mutated region of 57 of these types. If additional samples are available in the future, further examination as to whether this method can detect these remaining mutant alleles should be performed. Based on the above findings, the QP method appears to be an effective tool for screening *NPM1* mutations in AML cases.

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