Detection of Nucleophosmin 1 Mutations by Quantitative Real-Time Polymerase Chain Reaction Versus Capillary Electrophoresis

A Comparative Study

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• Context.—Nucleophosmin 1 (NPM1) is the most commonly mutated gene in acute myeloid leukemia. Detection of NPM1 mutations is useful for stratifying patients for therapy, predicting prognosis, and assessing for minimal residual disease. Several methods have been developed to rapidly detect NPM1 mutations in genomic DNA and/or messenger RNA specimens.

Objective.—To directly compare a quantitative real-time polymerase chain reaction (qPCR) assay with a widely used capillary electrophoresis assay for detecting NPM1 mutations.

Design.—We adopted and modified a qPCR assay designed to detect the 6 most common NPM1 mutations and performed the assay in parallel with capillary electrophoresis assay in 207 bone marrow aspirate or peripheral blood samples from patients with a range of hematolymphoid neoplasms.

The nucleophosmin 1 (NPM1) gene, located on band 5q35, is composed of 12 exons and encodes a nucleolar phosphoprotein that has many cellular functions. A major role of NPM1 is to function as a molecular chaperone for preribosomal proteins being transported between the nucleus and the cytoplasm. NPM1 also interacts with tumor suppressor proteins, such as p53, ARF, and IFR1, and interacts with regulators of hematopoiesis, such as HOX. NPM1 is one of the most commonly mutated genes in acute myeloid leukemia (AML), found in approximately one-third of adult cases. The frequency of this mutation is particularly high in AML with a normal karyotypic finding, in about 50% to 60% of diploid cases. Patients with NPM1-mutated AML commonly present with a high white blood cell count at time of diagnosis. The blasts in NPM1-mutated AML commonly exhibit monocytic differentiation and are often negative for CD34. Cases of AML with NPM1 mutation also have a distinct gene expression profile. Recent studies have associated the NPM1 mutation with a better prognosis in the absence of FLT3 mutation. These patients have better response to therapy, a higher complete remission rate, and longer survival. The 2008 World Health Organization classification has created a new provisional entity, AML with mutated NPM1, and recommends screening for this mutation in all patients with AML because of the potential clinical importance of the NPM1 mutation. Moreover, the NPM1 mutation is thought to be an early event in AML pathogenesis and is found to be very stable at relapse. Therefore, the presence of an NPM1 mutation is a potentially useful marker for assessing minimal residual disease (MRD) after therapy, especially in patients with diploid AML. More than 40 NPM1 mutations have been described to date. Almost all of these mutations are located in a mutational hot-spot region in exon 12, from positions 956 to 971, with very rare mutations also reported in exon 11. NPM1 mutations are entirely somatic, almost always heterozygous, and consist of insertions or deletions of short nucleotide stretches that cause a reading frame shift, destruction of the nucleolar localization signal, and creation of a nuclear export signal at the carboxy terminus. As a result, mutant protein loses its function and abnormally accumulates in the cytoplasm.

Results.—The qPCR assay demonstrated a higher analytical sensitivity than the capillary electrophoresis 1/1000 versus 1/40, respectively. The capillary electrophoresis assay generated 10 equivocal results that needed to be repeated, whereas the qPCR assay generated only 1 equivocal result. After test conditions were optimized, the qPCR and capillary electrophoresis methods produced 100% concordant results, 85 positive and 122 negative.

Conclusions.—Given the higher analytical sensitivity and specificity of the qPCR assay, that assay is less likely to generate equivocal results than the capillary electrophoresis assay. Moreover, the qPCR assay is quantitative, faster, cheaper, less prone to contamination, and well suited for monitoring minimal residual disease.

( Arch Pathol Lab Med. 2011;135:994–1000)
Three NPM1 mutations are most commonly observed in AML. These include the type A mutation that represents 75% to 80% of all NPM1 mutations, as well as type B and type D mutations, representing 10% and 5%, respectively. All other reported NPM1 mutations are rare.\textsuperscript{21}

A number of methods have been developed to rapidly detect NPM1 mutations in genomic DNA and/or mRNA specimens.\textsuperscript{22-27} The most widely adopted screening method, routinely used in our laboratory, is polymerase chain reaction (PCR) amplification of the mutational hot-spot region followed by capillary electrophoresis analysis. This approach yields semiquantitative results. Recently, several groups have designed quantitative real-time polymerase chain reaction (qPCR)-based assays for detecting single or multiple common NPM1 mutations in AML.\textsuperscript{23-27} These methods offer the advantage of reliable quantification of mutated NPM1 and, therefore, would be useful for providing a quantitative measurement of disease burden and for assessing MRD after therapy.\textsuperscript{25-27}

The clinical applications of these assays, however, and whether they are ready to replace conventional capillary electrophoresis assays are still being debated. In this study, we have assessed and validated a qPCR method to detect NPM1 mutations, and we tested in parallel a large number of cases comparing the qPCR assay with our routine capillary electrophoresis method.

**MATERIALS AND METHODS**

**Samples and DNA Preparation**

We randomly selected 207 bone marrow aspirate or peripheral blood specimens for which NPM1 mutation testing was ordered and residual DNA was available from the archives of the Molecular Diagnostics Laboratory at the University of Texas M. D. Anderson Cancer Center in Houston. These samples were accessioned from October 1, 2008, through June 30, 2009. For each case, the pathology reports and associated laboratory data were reviewed and the diagnosis was evaluated according to the 2008 World Health Organization criteria. Two cell lines, OCI-AML3 and HL60, were maintained in RPMI medium (Invitrogen, Carlsbad, California) supplemented with 5% fetal calf serum, 1% glutamine, and 100 U/mL penicillin in a 37°C incubator containing 5% carbon dioxide. Genomic DNA was extracted using the Autopure extractor (QIAGEN/Gentra, Valencia, California). The study was performed according to an approved laboratory protocol and was in accordance with the Declaration of Helsinki.

**NPM1 Mutation Detection by Capillary Electrophoresis Analysis**

Primers were designed to amplify the mutational hot spots spanning codons 956-971. The forward primer (5'-TGTTAAAGCCAGCGGCTAGT-GATGGTAACTATGCAAAGAC-3') and reverse primer (5'-CAGGAAACAGCTATGAGGGTGGAGGATGTCAC-3') contained 1 M13 tag, and the expected amplicon size was 268 base pairs. Reaction mixtures of 50 µL contained 1 mM deoxyribonucleoside triphosphate, 2 mM magnesium chloride, 200 nM primers, and 1.5 U Tli Polymerase. Two hundred nanograms of genomic DNA was amplified using the following PCR conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C. A final extension step at 72°C for 7 minutes was then performed.

Fluorescently labeled PCR products, without prior purification, were diluted 1:10 in deionized water. For capillary electrophoresis, 1 µL fluorescently labeled PCR product was combined with 19.8 µL of deionized formamide (AMRESCO Inc., Solon, Ohio) and 0.2 µL of GS500 ROX-size standard (Applied Biosystems, Foster City, California). Samples were heat-denatured for 5 minutes at 95°C and rapidly chilled on ice for 5 minutes before loading onto an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The resulting data were analyzed by fragment size using GeneMapper software (Applied Biosystems). The analytical sensitivity and specificity of the assay were determined by serial dilution studies using the OCI-AML3 cell line, which contains an NPM1 type A mutation DNA, mixed with the HL60 cell line, containing wild-type NPM1.

**NPM1 Mutation Detection by Quantitative Real-Time PCR**

A multiplex qPCR assay was designed by modifying the method reported by Gorello et al.\textsuperscript{26} As shown in Figure 1, 6 mutation-specific forward primers, 1 common reverse primer, and a common probe were designed for the detection of the 6 most common types of NPM1 mutations (A, B, D, E, G, and H). A reaction mixture of 25 µL contained 1× TaqMan Universal Master Mix with AmpErase uracil N-glycosylase (Applied Biosystems), 0.4 µM primers, and 0.2 µM 6-carboxyfluorescein-labeled probe. After activation of uracil N-glycosylase at 50°C for 2 minutes, 500 ng of genomic DNA was amplified with the following PCR conditions: an initial 10-minute denaturation at 95°C, followed by 42 cycles of 15 seconds at 95°C, and 1 minute at 61°C. Real-time analysis was performed on the ABI PRISM 7900 Sequence Detection System containing a 96-well thermal cycler (Applied Biosystems). All samples were tested in duplicate. To assess the quantity and quality of DNA, the ribonuclease P gene (TaqMan RNase P Detection Reagents Kit; Applied Biosystems) was used as an internal control and amplified for each sample. Standard curves for NPM1 and ribonuclease P were generated by amplifying a 10-fold serial dilution of OCI-AML3 DNA (NPM1 type A mutation) into HL60 DNA (wild-type NPM1).

**NPM1 Mutation Detection by Sanger Sequencing**

Primers were designed within exon 12 using Primer3 freeware (Version 0.4.0; http://frodo.wi.mit.edu/primer3; Accessed April 8, 2010). The predicted wild-type amplicon size is 168 base pairs. The forward primer (5'- GATGTCATATGAAGTGTTGTGGTTC-3') was labeled with 6-carboxyfluorescein, and the reverse primer (5'-GAGAAGCAGCTATGAGGGTGGAGGATGTCAC-3') was not labeled. A reaction mixture of 50 µL contained 1 mM deoxyribonucleoside triphosphate, 2 mM magnesium chloride, 200 nM primers, and 1.5 U Tli Polymerase. Two hundred nanograms of genomic DNA was amplified with the following PCR conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C. A final extension step at 72°C for 7 minutes was also performed.

The PCR products were purified (Agencourt Ampure; Beckman Coulter Genomics, Brea, California) before being loaded onto an ethidium bromide-stained agarose gel. Fluorescently labeled cycle sequencing was performed using the dye-terminator method and a multipicapillary sequencer (ABI 3130 Genetic Analyzer; PE Biosystems, Foster City, California) according to the manufacturer's protocol (BigDye Terminator v1.1 Cycle Sequencing Kit; PE Biosystems). Briefly, reaction tubes (total volume 20 µL) containing 100 ng of PCR products, 3.2 pmol of either the forward or reverse M13 primer, and 6 µL of the sequencing mixture, were placed in a DNA thermal cycler and amplified at 96°C for 1 minute, 25 cycles at 96°C for 10 seconds, 58°C for 5 seconds, 60°C for 4 minutes, and final hold at 4°C. Sequence reactions were purified using the QIAGEN DyeEx purification kit (QIAGEN, Valencia, California) per the manufacturer's protocol. The resulting data were analyzed by SeqScape software (Version 2.6; PE Biosystems).

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**Arch Pathol Lab Med—Vol 135, August 2011**

**NPM1 Mutation by qPCR and Capillary Electrophoresis—Barakat et al**
In our clinical laboratory, we currently detect NPM1 mutation by using a capillary electrophoresis–based assay. The wild-type sequence generates a 168–base pair fragment on the electropherogram. All known exon 12 mutations will produce peaks of a larger size. Because all mutations are heterozygous, the wild-type peak is also used as an internal control for successful DNA extraction and PCR amplification. We developed this qPCR assay by modifying the method reported by Gorello et al,26 including the replacement of 2 original reverse primers with a single common reverse primer (Figure 1) and optimizing a multiplex reaction for all primer sets used to detect 6 NPM1 mutations in a single tube. With the qPCR assay, a wild-type sequence will not amplify by PCR. Hence, any amplification indicates the existence of a mutation. Negative results are validated by coamplification of the ribonuclease P gene.

To determine the analytical sensitivity and specificity of the qPCR assay and to compare the results with capillary electrophoresis analysis, we serially diluted the OC1-AML3 cell line DNA into the wild-type NPM1 HL60 cell DNA. Figure 2, A through C, and Figure 3, A and B, show the results of representative runs. Using the qPCR method, we were able to reproducibly detect NPM1 mutation in the 1:10 and 1:1000 dilutions but not always in the 1:10 000 dilution. By contrast, the sensitivity of our routine capillary electrophoresis–based method was 1:40.

We then compared the capability of 2 methods in detecting NPM1 mutations in 207 clinical samples from patients with hematolymphoid malignancies. The diagnoses of these patients, according to the World Health Organization system, were as follows: AML (56%; n = 116), myelodysplastic syndrome (17%; n = 35), myeloproliferative neoplasm (10%; n = 21), and other hematolymphoid neoplasms (17%; n = 35), including lymphomas, acute lymphoblastic leukemia, and chronic lymphocytic leukemia. The male to female ratio in this study cohort was 1.2. The patients ranged in age from 5 to 88 years.

Figure 1. Design of primers and probe for the quantitative real-time polymerase chain reaction–based NPM1 mutation assay. Six mutation-specific forward primers, 1 common reverse primer, and a common probe were designed for the detection of the 6 most common types (A, B, D, E, G, and H) of NPM1 mutations on exon 12.

Figure 2. Determination of analytical sensitivity of the capillary electrophoresis method by a serial-dilution study using DNA of the OC1-AML3 cell line, which contains an NPM1 type A mutation mixed with the DNA of the HL60 cell line, containing a wild-type NPM1. A–C, Results of serial dilutions.
Results showed 85 cases were positive for the NPM1 mutation in this study cohort, including 81 (95%) adult cases of AML and 4 adults with high-risk myelodysplastic syndrome. No NPM1 mutation was identified in the pediatric cases. Among the cases with NPM1 mutations, 81 (95%) had a diploid karyotype, and 4 cases (5%) had abnormal cytogenetics, including trisomy 8, del(16q), add(8)(q22), or del(5)(q15q33).

All 207 samples were examined in a parallel fashion by qPCR and capillary electrophoresis analysis. On finishing the first round of test runs, 196 specimens (95%) showed concordant results by both methods, and 11 cases (5%) were discordant, including 1 case that was questionably positive by qPCR only and 10 cases that were equivocal by capillary electrophoresis but negative by qPCR assay.

Further investigation showed that the case that was positive only by qPCR had a positive result in only 1 of 2 duplicates (Figure 4, A through C). In our routine practice for the reporting of clinical test results, this discordance between duplicates would have led to the same patient sample being retested. Repeat qPCR for this sample showed negative results in both duplicates supporting the interpretation that the initial qPCR result was falsely positive. By the shape of the amplification

Figure 3. Determination of analytical sensitivity of the quantitative real-time PCR (qPCR) assay by a serial dilution study using DNA of the OC1-AML3 cell line, which contains an NPM1 type A mutation mixed with the DNA of HL60 cell line containing wild-type NPM1. A, Amplification plot of the serial dilutions. Cycle, the number of PCR cycles; ΔRn, the magnitude of the signal generated by the PCR. B, An example of the standard curve. Quantity, relative quantity of PCR product; Ct, threshold cycle.

Figure 4. A case that had a negative finding for the NPM1 mutation by capillary electrophoresis but was questionably positive by quantitative real-time PCR (qPCR). A, The initial questionably positive qPCR result. B, Repeat qPCR result was clearly negative. A and B, Cycle, the number of PCR cycles; ΔRn, the magnitude of the signal generated by the PCR. C, Negative result by capillary electrophoresis on the same sample. sz, size of the amplicon; ar, area under the peak.
The false-positive result was most likely due to a tiny air bubble in the tube.

Given the nature of the capillary electrophoresis assay, sometimes equivocal results are generated for technical reasons. As shown in the example (Figure 5), when the mutation level is low or the background peaks are high because of overloading or contamination, it is difficult to determine whether a peak represents the existence of a mutant sequence or is a nonspecific finding that is part of the background. Repeats with modifications of the loading conditions can usually solve these issues. In another case with an equivocal result, the mutation peak showed up only in one duplicate. One possible explanation is contamination caused by carryover from another patient sample. In this particular case, there was another sample that was positive for NPM1 mutation in the same run, providing a possible source of the contamination.

After optimizing the conditions, all samples with equivocal results by capillary electrophoresis analysis had negative results on repeat analysis and were in agreement with the qPCR results (Table 1). All mutation-specific primers in this assay were proven to be highly specific because there was no amplification of the wild-type sequence in either the serial dilution study or the following validation study using clinical samples. Sanger sequence analysis was performed in 26 cases for the purpose of confirmation. These included the 11 initially discordant samples, which were all wild type by Sanger sequencing, and another 15 mutated cases that were also confirmed. However, the analytical sensitivity of the mutation detection by Sanger sequencing is less than that of capillary electrophoresis or qPCR methods and, therefore, not an ideal choice to confirm negative results.

Table 2 compares the technical and economic aspects of the qPCR assay and capillary electrophoresis analysis. The qPCR method has substantially higher analytical sensitivity and is more quantitative, faster, and less expensive. The qPCR method and is also a closed-tube assay. Although there is a potential that this assay may miss the less frequent mutation types, no other NPM1 mutations were detected in these 207 cases analyzed, indicating that the rarely described NPM1 mutations are truly rare.

**COMMENT**

NPM1 mutation status in patients with AML is clinically important for the purposes of determining prognosis and stratifying patients for therapeutic protocols. As a result, it is recommended that NPM1 mutation status be screened routinely in patients with AML, particularly in those patients with diploid cytogenetics and those with some high-risk myelodysplastic syndromes. Although Sanger sequencing is applicable for most types of mutation testing, Sanger sequencing is of limited analytical sensitivity, is more labor-intensive, and has a relatively higher economic cost and, therefore, is not an ideal method for routine high-volume screening. Capillary electrophoresis analysis is currently the most widely adopted method for detecting NPM1 mutations. A great advantage with this method is that a single PCR, followed by capillary electrophoresis, is able to detect most of the known NPM1 mutations in exon 12. However, there are limitations associated with capillary electrophoresis. Most important, it is a semiquantitative method with limited

**Figure 5.** A representative result by capillary electrophoresis that was initially equivocal for the NPM1 mutation but was a clearly negative result by quantitative real-time PCR (qPCR). A, The initial, questionably positive capillary electrophoresis result. B, Repeat capillary electrophoresis result was clearly negative. Abbreviations [A and B]: sz, size of the amplicon; ar, area under the peak. C, Negative result by qPCR on the same sample. Abbreviations: cycle, the number of PCR cycles; ΔRn, the magnitude of the signal generated by PCR.
analytical sensitivity, and capillary electrophoresis is prone to generate equivocal results when the tumor cell count is low. In addition, capillary electrophoresis is associated with a higher cost in instruments and reagents. Several alternative methods have been reported recently for rapid and accurate detection of one or several of the most frequent types of NPM1 mutations, including qPCR approaches. There is no direct comparison of qPCR with capillary electrophoresis analysis for detecting NPM1 mutations available in the literature.

In this study, we developed and validated a qPCR assay for detecting the common NPM1 mutations. We simplified the original design by designing a common reverse primer for all 6 reactions and improved its efficiency by multiplexing all reactions into a single tube. We believe that this qPCR method has many advantages. First, qPCR has a higher analytical sensitivity than our standard NPM1 detection method using capillary electrophoresis analysis (1:1000 for qPCR versus 1:40 for capillary electrophoresis). Second, qPCR results are quantitative, allowing the use of the qPCR assay to follow patients by detection of MRD.5–27 Third, the qPCR method requires fewer steps and, therefore, has a shorter turnaround time. In capillary electrophoresis analysis, after the PCR is completed, gel loading and analysis are required. Fourth, the extra step required for capillary electrophoresis also increases the risk of contamination, which is minimized when using the qPCR assay because it is a closed-tube assay, and with uracil N-glycosylase technology, the possibility of cross-contamination is essentially eliminated. Lastly, the qPCR approach is less expensive than capillary electrophoresis analysis, which requires 2 instruments and is more labor intensive.

We validated this qPCR assay by testing, in parallel, 207 clinical patient samples. These samples were selected for study because NPM1 mutation testing had been ordered as part of the patient workup, and residual DNA was available for this study, but otherwise, the samples were chosen randomly. Once the testing conditions were optimized, the qPCR and capillary electrophoresis methods produced 100% concordant results. The analytic specificity of the qPCR assay was 100% in this study, with no amplification of the wild-type sequence. These results support the interpretation that this qPCR method and capillary electrophoresis analysis are essentially equivalent for detecting NPM1 mutation. Only one sample analyzed had an unclear result by qPCR and needed repeat analysis, whereas 10 samples analyzed by capillary electrophoresis analysis initially had equivocal results. In our opinion, therefore, the qPCR assay is more reproducible. We believe that the equivocal results by capillary electrophoresis analysis can be explained by the sample loading conditions needing to be adjusted to produce an optimal electropherogram output. In this study, simple repeat analysis of the cases with discordant or equivocal results resolved these discrepancies.

A major concern with using the qPCR method is the limitation that not all exon 12 mutations can be detected. The qPCR assay we used detects 6 of the most common NPM1 mutations, representing at least 95% of all NPM1 mutations reported.10 Because the NPM1 mutation in the absence of the FLT3 mutation is associated with a good prognosis, missing a mutation would potentially subject the patient to an unnecessary high-risk procedure, such as a bone marrow transplant. Originally, we expected that at least a few cases in our 207 study cohort would have negative results by qPCR but positive results by capillary electrophoresis analysis, attributable to a rare NPM1 mutation. Because this did not occur, it appears that the 6 NPM1 mutations detected by qPCR may represent more than 95% of all cases, at least in our patient population, ameliorating the concern about missing rare NPM1 mutations using this qPCR method.

In conclusion, we compared a qPCR method with our routinely used capillary electrophoresis analysis to detect NPM1 mutations. The results show that these 2 methods are essentially equivalent in detecting NPM1 mutations in a large number of patient specimens. The qPCR method, however, has a number of advantages. Most important, qPCR has a higher analytic sensitivity than capillary electrophoresis analysis and yields quantitative results. Therefore, qPCR is better suited for following patients after therapy for MRD.5–27 The qPCR approach also requires one less step and, by removing that step, minimizes the potential for contamination, improves turnaround time, and reduces the manual labor involved in NPM1 mutational analysis. One potential drawback to the qPCR approach is that the assay is designed to detect only the 6 most common NPM1 mutations and can, therefore, theoretically “miss” rare mutations. However, our experience suggests that NPM1 mutations other than the 6 assessed by this qPCR assay are truly rare, perhaps representing less than 1% of all NPM1 mutations and are, therefore, not a practical concern in routine screening.

Table 2. Detailed Comparison of Quantitative Real-Time PCR (qPCR) and Capillary Electrophoresis Methods in NPM1 Mutation Detection

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<tr>
<th>qPCR</th>
<th>Capillary Electrophoresis</th>
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<tr>
<td>Analytical sensitivity</td>
<td>At least 1/1000</td>
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<td>Number of steps postextraction</td>
<td>1 step</td>
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<td>Turnaround time</td>
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<td>Risk of contamination</td>
<td>Lower</td>
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<td>Result nature</td>
<td>Quantitative</td>
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<td>Cost</td>
<td>Less expensive</td>
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<td>Use for MRD</td>
<td>Applicable</td>
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<tr>
<td>No. of mutations detected</td>
<td>6 (&gt;95% of known mutations)</td>
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Abbreviations: MRD, minimal residual disease; NPM1, nucleophosmin 1.

References


