Technical Advance

An Allele-Specific RT-PCR Assay to Detect Type A Mutation of the Nucleophosmin-1 Gene in Acute Myeloid Leukemia

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Nucleophosmin-1 (NPM1) mutations represent the most frequent gene alteration in acute myeloid leukemia (AML). The most common NPM1 mutation type, accounting for 75 to 80% of cases, is referred to as mutation A (NPM1-mutA). These NPM1 alterations have been shown to possess prognostic significance because they appear to identify patients who will benefit from chemotherapy. Given the high prevalence and stability of these mutations over the course of disease, NPM1 mutations may serve as ideal targets for minimal residual disease (MRD) assessment in AML. Current detection methods are costly, require sophisticated equipment, and are often not sufficiently sensitive. We report here an allele-specific (ASO)-RT-PCR assay that enables rapid and sensitive detection of NPM1-mutA. A semi-nested ASO-PCR method was also designed to increase the sensitivity of our assay for the monitoring of MRD. We analyzed bone marrow cells collected from 52 patients with AML at presentation. NPM1-mutA was detected in leukemic cells from 21 patients. Assay specificity was confirmed by capillary electrophoresis and DNA sequencing. ASO-RT-PCR and semi-nested ASO-PCR assays could detect NPM1-mutA with sensitivities of 10^{-2} and 10^{-5} , respectively. Results obtained here verify that our ASO-RT-PCR assay is a specific and sensitive method for the routine screening of NPM1-mutA, as well as for MRD monitoring of AML patients

with this alteration. This method is convenient and easily applicable in countries with limited resources and no access to real-time quantitative PCR-based technologies. (J Mol Diagn 2008; 10:212–216; DOI: 10.2353/jmoldx.2008.070166)

Nucleophosmin-1 (NPM1) is a nucleocytoplasmic shuttling protein that plays a key role in a variety of cellular processes including promotion of ribosome biogenesis, maintenance of genomic stability, regulation of transcription, and modulation of tumor-suppressor transcription factors.¹ The NPM1 gene is one of the most frequent targets of genetic alterations in hematopoietic tumors as it has been shown to be involved in several gene fusions resulting from chromosome translocations and in other subtle mutations detected in lymphomas and acute leukemia.²

In early 2005, Falini et al³ reported that NPM1 gene mutations, mainly consisting of small nucleotide insertion/deletions, occur frequently in acute myeloid leukemia (AML) and are strongly associated with normal karyotype. Over 40 different types of mutations in the NPM1 locus have been described so far, which result in the formation of different mutant proteins. These alterations consist of the insertion/deletion of short nucleotide stretches (4 or 10 bp) at positions 956 through 971 in NPM1 exon 12 that lead to an open reading frame shift resulting in different protein variants containing novel C terminus portions.^{3–5} The most common NPM1 mutation type, accounting for 75% to 80% of cases, is referred to as mutation A (NPM1-mutA)³ and consists of a duplication of a TCTG tetranucleotide at position 956 to 959 of the reference sequence (GenBank accession number NM_002520).

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Table 1. Primers Used for ASO-RT-PCR, Semi-nested ASO-PCR, Sequencing, and Fragment Analysis

Name	Sequence	Dye	PCR product (size)	Application
NPM-A	5'-CCAAGAGGCTATTCAAGATCTCTCTC-3'		NPM1-mut-A ex 12 (320 bp)	ASO-RT-PCR NPM1-mutA
NPM-AN	5'-CAAGAGGCTATTCAAGATCTCTGTCTG-3'		NPM1-mut-A ex 12 (319 bp)	Semi-nested ASO-PCR NPM1-mutA
NM-F2	5'-ATCAATTATGTGAAGAATTGCTTAC-3'		NPM1 ex 12 (349/353 bp)	NPM1 ex 12 sequencing
NPM-REV-6	5'-ACCATTTCCATGTCTGAGCACC-3'			ASO-RT-PCR NPM1-mutA, semi-nested ASO-PCR NPM1-mutA and sequencing
(D4)NPM-REV-6	5'-ACCATTTCCATGTCTGAGCACC-3'	D4		NPM1-mut (fragment analysis)
ABL-A2B-5'	5'-GCATCTGACTTTGAGCCTCAG-3'		ABL (258 bp)	Internal PCR control
ABL-A3E-3'	5'-TGACTGGCGTGATGTAGTTGCTT-3'			Internal PCR control

To date, *NPM1* gene mutations represent the most frequent known genetic abnormality in AML. Moreover, these alterations have been shown to carry prognostic significance because they seem to identify patients with better response to chemotherapy. As a consequence, the analysis of *NPM1* mutational status is now recommended for inclusion in the genetic routine characterization of AML.⁶⁻¹¹ Finally, given their high prevalence and stability over the course of the disease, *NPM1* mutations may serve as an ideal target for minimal residual disease (MRD) assessment, particularly for patients with AML-normal karyotype.⁶

Several protocols and techniques have been developed for the detection of *NPM1* mutations. These include DNA sequencing, a real-time quantitative PCR assay,⁶ denaturing high-performance liquid chromatography,⁷ capillary electrophoresis,⁸ and locked nucleic acid-mediated PCR clamping.⁹ Although these methods allow high specificity to assess *NPM1* mutational status at diagnosis, most have drawbacks including an elevated cost and requirement of sophisticated equipment and, except for RQ-PCR and locked nucleic acid-PCR clamping, poor sensitivity. To date, a highly specific, low-cost and sensitive method for the detection of *NPM1* mutations using non-quantitative PCR has not been developed. In this study we report an allele-specific (ASO)-RT-PCR assay that enables rapid and sensitive detection of the most common *NPM1* mutation. This test also seems suitable for assessment of response and longitudinal molecular monitoring of patients with AML harboring *NPM1-mutA*.

Materials and Methods

Patients and Samples

After informed consent, bone marrow cells were collected at presentation from 52 patients with AML diagnosed at the Institute of Hematology, Department of Biopathology of the University Tor Vergata of Rome. Total RNA was extracted from Ficoll-Hypaque-isolated mononuclear cells using the method of Chomczynski and Sacchi.¹² RNA was reverse-transcribed using random hexamer primers as previously described.¹³ RNA was chosen as starting material (instead of DNA) because of the need to routinely amplify fusion genes (*AML1/ETO*, *BCR/ABL*

[*Abelson* gene], *CBFbeta/MYH11*, and others) in the diagnostic workup of AML, which can only be feasible using cDNA.

Three cDNA aliquots were used for ASO-RT-PCR and semi-nested ASO-PCR, sequencing, and capillary electrophoresis experiments, respectively.

ASO-RT-PCR

With the aim of analyzing the presence of type A mutation in *NPM1* exon 12, we used an ASO-RT-PCR strategy. A forward primer (NPM-A; Table 1) was designed to specifically amplify *NPM1* exon 12 only if the mutA was present. This primer in fact contains an intentional mismatch at the third nucleotide from the 3' end to improve specificity. The amplified region includes the insertion of a TCTG tetranucleotide at positions 956 to 959 of the reference sequence. In this region *NPM1* and its seven pseudogenes¹⁴ are highly homologous. Therefore, to rule out the amplification of pseudogenes, we used a previously described reverse primer (NPM-REV-6) excluding the amplification of pseudogenes.⁸

A 320-bp fragment was amplified from 1 μ l of cDNA in a total volume of 25 μ l of the reaction mixture containing 10 pmol of each primer, NPM-A and NPM-REV-6, 1 $\times$ PCR buffer, 2.5 mmol/L MgCl₂, 5 mmol/L deoxynucleoside-5'-triphosphates, and 0.7 U of Taq polymerase (Applied Biosystems, Foster City, CA).

Preheating of the mixture at 95°C for 7 minutes was followed by 35 cycles of 30 seconds at 95°C, 45 seconds at 67°C, and 45 seconds at 72°C. A final extension of 7 minutes was performed at 72°C on a Gene Amp PCR System 2400 (Perkin Elmer, Emeryville, CA). PCR products were visualized by electrophoresis on 2% (w/v) agarose gel. As internal PCR control, we used *ABL* amplification with the same ASO-RT-PCR conditions but using the primers ABL-A2B-5' and ABL-A3E-3'.

To assess the detection threshold of our assay, we performed serial dilution experiments using RNA obtained from an AML patient's marrow with >95% leukemic cells and harboring the *NPM1-mutA*, with RNA extracted from the bone marrow of a patient with *NPM1-w/t* (wild-type) AML (dilution ratios *NPM1-mutA/NPM1-w/t*: 1/10, 1/10², 1/10³, 1/10⁴, 1/10⁵, and 1/10⁶).

Semi-Nested ASO-PCR

A semi-nested ASO-PCR strategy was designed to increase the sensitivity of our assay for the monitoring of MRD. For this purpose, we designed an internal specific forward primer for NPM1-mutA (NPM-AN). A 0.5- μ l aliquot of the first step ASO-RT-PCR was amplified in a total volume of 25 μ l of the reaction mixture containing 10 pmol of each primer, NPM-AN and NPM-REV-6, 1 $\times$ PCR buffer, 2.5 mmol/L MgCl₂, 5 mmol/L deoxynucleoside-5'-triphosphates, and 0.7 U of Taq polymerase (Applied Biosystems). Preheating of the mixture at 95°C for 7 minutes was followed by 35 cycles of 30 seconds at 95°C, 45 seconds at 67°C, and 45 seconds at 72°C. A final extension of 7 minutes was performed at 72°C on a Gene Amp PCR System 2400 (Perkin Elmer). PCR products were visualized by electrophoresis on a 2% (w/v) agarose gel.

Capillary Electrophoresis of NPM1 RT-PCR Products

To independently validate the specificity of the ASO-RT-PCR and NPM1-mutA assay, we analyzed in parallel all diagnostic samples by DNA sequencing (see below) as well as by a previously developed RT-PCR assay followed by capillary electrophoresis. The primers and PCR conditions to amplify NPM1 exon 12 have been described previously.⁸ For capillary electrophoresis, sample dilutions of 1:20 and 1:400 were prepared in a total volume of 20 μ l of Sample Loading Solution (SLS) (CEQTM SLS p/n 608082; Beckman Coulter, Fullerton, CA) containing 0.25 μ l of CEQ 600 size standard mixture (CEQTM DNA Size Standard Kit-600 p/n 608095; Beckman Coulter). Samples were loaded in 96-well plates and covered with mineral oil. The amplified products were separated with a capillary electrophoresis-based system (CEQ 8000 Genetic Analysis system; Beckman Coulter) using the Frag Test default run method.

Figure 1. A: ASO-RT-PCR. An amplification band of 320 bp containing the NPM1-mutA is visualized in three patient samples (lanes 1, 2, and 3), whereas no amplification signal is visible in three patient samples with germline NPM1 (lanes 4, 5, and 6). **B:** successful amplification of the ABL gene used in all cases as internal PCR control. M, molecular weight marker.

Sequencing Analysis of NPM1 Exon12

All samples analyzed were sequenced to confirm the results obtained by ASO-RT-PCR. cDNAs were amplified using the primers NPM-REV-6 and NM-F2 (see Table 1) and PCR conditions reported elsewhere.⁸ The amplified products were purified using a QIAquick PCR extraction kit (Qiagen Inc., Chatsworth, CA). PCR products were cloned into a plasmid vector pCR 2.1-TOPO and then transformed into chemically competent TOP10F' One Shot *Escherichia coli* (TOPO TA Cloning; Invitrogen, Carlsbad, CA). For each sample, at least five recombinant colonies were selected, and plasmid DNA was prepared using a QIAprep Spin Miniprep Kit (Qiagen Inc.) and sequenced (CEQ 8000 Genetic Analysis system; Beckman Coulter). Sequences were compared with the NPM1-w/t cDNA (GenBank accession number NM_002520). Numbering of nucleotide positions refers to the coding sequence. Primer sequences used for ASO-RT-PCR, semi-nested ASO-PCR, sequencing, and fragment analysis are shown in Table 1.

Results and Discussion

Using our ASO-RT-PCR assay, a fragment of the expected size of 320 bp presumably containing the NPM1-

Figure 2. Electropherograms of an NPM1-mut (A) and an NPM1-w/t (B) sample.

Figure 3. Sequence traces showing the NPM1-mutA (**A**) and NPM1-w/t (**B**) pattern. The mutant sequence pattern in **A** contains the TCTG duplication.

mutA was amplified in 21 of the 52 patients, whereas no amplification bands were detected in the remaining 31 cases. The *ABL* fragment of 258 bp used as internal control was successfully amplified in all patients. The results of the ASO-RT-PCR assay in some representative cases are shown in Figure 1.

To validate the assay, all samples were further analyzed by a previously reported RT-PCR method followed by capillary electrophoresis.⁸ The labeled fragment sizes corresponding to NPM1-mut and NPM1-w/t were 353 bp and 349 bp, respectively (Figure 2, A and B). This comparison showed concordance of results in 100% of cases by confirming in all instances NPM1-mutA and NPM1-w/t cases. The specificity of our assay test was finally confirmed by sequencing the PCR product in all 52 samples. The results of sequencing analysis in two representative samples with NPM1-mutA and NPM1-w/t are shown in Figure 3, A and B.

Figure 4. ASO-RT-PCR and semi-nested ASO-PCR of serial dilutions of NPM1-mutA. **Lanes 1, 2, 3, and 4** correspond to the amplification by ASO-RT-PCR of 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} dilutions, respectively. **Lanes 5, 6, 7, 8, 9, and 10** correspond to the amplification by semi-nested ASO-PCR of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilutions. **Lanes 11 and 12** correspond to the undiluted positive control and negative control, respectively. M, molecular weight marker.

Figure 5. Results of semi-nested ASO-PCR analysis to amplify NPM1-mutA for MRD detection in four patients (**a**, **b**, **c**, and **d**) tested at diagnosis and during follow-up. The figure represents NPM1-mutA (■) and NPM1-w/t (□) results of the semi-nested ASO-PCR assay.

To establish the sensitivity of our ASO-RT-PCR diagnostic assay and that of a semi-nested ASO-PCR method to be used for monitoring studies, serial dilutions from positive cDNA NPM1-mutA (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}) were performed. The ASO-RT-PCR and the semi-nested ASO-PCR showed a sensitivity of 1:100 and 1:100,000, respectively (Figure 4).

To assess MRD during patient follow-up, we analyzed longitudinally at different time points marrow samples from four AML patients who achieved hematological remission after chemotherapy. Using the semi-nested ASO-PCR, all samples tested PCR negative during remission after one, four, and eight months of therapy. One patient subsequently relapsed after autologous bone marrow transplantation. Unfortunately, no sample close to the relapse one was available for the analysis to verify whether the assay was able to predict relapse at the molecular level (Figure 5). The prospective analysis of a larger number of cases is needed to assess the clinical relevance of sequential PCR monitoring using this test.

In conclusion, the ASO-RT-PCR assay described here represents a specific and sensitive method for routine screening of NPM1-mutA, and a suitable test for minimal residual disease monitoring of AML patients with this alteration. Prospective longitudinal studies in large patient series are needed to investigate the clinical relevance of this test to assess response to therapy and to predict disease relapse. Finally, our method seems convenient and easily applicable in countries with limited resources and no access to RQ-PCR-based technologies.

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