Nucleophosmin (NPM1) is a multifunctional, highly conserved protein found most frequently in nucleoli. NPM1 acts as a molecular chaperone (1) and is thought to participate in preribosome maturation and centrosome duplication (2). In addition, it has been implicated in the regulation of the Arf-p53 tumor suppressor pathway (3, 4).

NPM1 mutations have recently been reported to occur at high frequency in acute myeloid leukemia (AML), for which they currently represent the most common detectable genetic lesion (~35% of cases). This abnormality is strongly associated with normal-karyotype AML and has never been detected in AMLs bearing major cytogenetic abnormalities. It has not been observed in other hematopoietic tumors (5). Two main types of mutations have been described to date. The first and most frequent consists of a 4-nucleotide (nt) insertion (YW1G; YUPAC code) downstream from nucleotide 959; the second is deletion of a GGAGG sequence at positions 965 through 969 and substitution with 9 extra nt (GenBank accession no. NM_002520). Both mutations lead to aberrant cytoplasmic localization of NPM1 as shown after immunostaining with anti-NPM1 monoclonal antibodies. In addition to their high frequency and clustering with normal karyotype, NPM1 mutations may identify a subset of AMLs with distinct response to therapy (5). Together, these findings may have repercussions in AML classification and suggest that analysis of NPM1 mutational status should integrate modern genetic characterization of AML. We describe here a rapid and reproducible method for screening NPM1 mutations by reverse transcription-PCR followed by denaturing HPLC (DHPLC).

Bone marrow samples showing at least 70% bone marrow infiltration by leukemic cells were collected at diagnosis from 56 patients with newly diagnosed AML observed at the Department of Biopathology at the University Tor Vergata (Rome). According to the French-American–British classification (6, 7), the following subtypes were included in the study population: 6 M0, 9 M1, 14 M2, 15 M4, 6 M5a, 4 M5b, and 2 M6. Fifty-three of 56 patients were evaluable for karyotype. Of these, 10 patients had favorable karyotypes [5 with t(8;21) and 5 with inv(16)], 30 patients had intermediate karyotypes [20 with
normal karyotype (6 + 8), and 4 cases with other intermediate lesions], and 13 patients had unfavorable karyotypes (7 with deletion of chromosomes 5 and/or 7, and 6 cases with complex karyotypes).

Written informed consent was obtained from all patients. Total RNA was extracted from Ficoll-Hypaque-isolated leukemic blasts by the method of Chomczynski and Sacchi (8). RNA (1 μg) was reverse-transcribed to cDNA by use of random examer primers as described previously in the BIOMED-1 Concerted Action protocol (9).

cDNA (2 μL) was amplified in a total volume of 50 μL. The reaction mixture contained 2.0 mM MgCl₂, 200 μM of each deoxynucleotide triphosphate, 1× PCR buffer, 1.5 U of Taq-Gold DNA polymerase (Perkin-Elmer Cetus), and 10 pmol each of the forward (5’-CTCTTCCCAAAGTGGAAGGCAA-3’) and reverse (5’-ACCAATTCATGTCTGAGCACC-3’) primers. A G-to-C mutation was introduced in the reverse primer to reduce amplification of NPM1 pseudogenes. After the mixture was preheated at 94 °C for 7 min, it was subjected to 30 cycles of 45 s at 55 °C, 30 s at 72 °C, and 30 s at 94 °C. A final extension of 5 min was carried out at 72 °C on a GeneAmp PCR System 2400 (Perkin-Elmer). DHPLC was carried out on a WAVE DNA fragment analysis system (TransgenomicTM) equipped with a DNASe⁰ column (Transgenomic). DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mol/L triethylamine acetate buffer (TEAA; Transgenomic) at a constant flow rate. The reversed-phase gradient was formed by mixing buffer A (0.1 mol/L TEAA, pH 7.0) and buffer B (250 mL/L acetonitrile in 0.1 mol/L TEAA, pH 7.0). Oven temperature for optimum separation of heteroduplex molecules was deduced from 55 °C, 30 s at 72 °C, and 30 s at 94 °C. A final extension of 5 min was carried out at 72 °C on a GeneAmp PCR System 2400 (Perkin-Elmer). DHPLC was carried out on a WAVE DNA fragment analysis system (TransgenomicTM) equipped with a DNASe⁰ column (Transgenomic). DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mol/L triethylamine acetate buffer (TEAA; Transgenomic) at a constant flow rate. The reversed-phase gradient was formed by mixing buffer A (0.1 mol/L TEAA, pH 7.0) and buffer B (250 mL/L acetonitrile in 0.1 mol/L TEAA, pH 7.0). Oven temperature for optimum separation of heteroduplex molecules was deduced from 55 °C, 30 s at 72 °C, and 30 s at 94 °C. A final extension of 5 min was carried out at 72 °C on a GeneAmp PCR System 2400 (Perkin-Elmer). DHPLC was carried out on a WAVE DNA fragment analysis system (TransgenomicTM) equipped with a DNASe⁰ column (Transgenomic). DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mol/L triethylamine acetate buffer (TEAA; Transgenomic) at a constant flow rate. The reversed-phase gradient was formed by mixing buffer A (0.1 mol/L TEAA, pH 7.0) and buffer B (250 mL/L acetonitrile in 0.1 mol/L TEAA, pH 7.0). Oven temperature for optimum separation of heteroduplex molecules was deduced from 55 °C, 30 s at 72 °C, and 30 s at 94 °C. A final extension of 5 min was carried out at 72 °C on a GeneAmp PCR System 2400 (Perkin-Elmer). DHPLC was carried out on a WAVE DNA fragment analysis system (TransgenomicTM) equipped with a DNASe⁰ column (Transgenomic). DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mol/L triethylamine acetate buffer (TEAA; Transgenomic) at a constant flow rate. The reversed-phase gradient was formed by mixing buffer A (0.1 mol/L TEAA, pH 7.0) and buffer B (250 mL/L acetonitrile in 0.1 mol/L TEAA, pH 7.0). Oven temperature for optimum separation of heteroduplex molecules was deduced from 55 °C, 30 s at 72 °C, and 30 s at 94 °C. A final extension of 5 min was carried out at 72 °C on a GeneAmp PCR System 2400 (Perkin-Elmer). DHPLC was carried out on a WAVE DNA fragment analysis system (TransgenomicTM) equipped with a DNASe⁰ column (Transgenomic). DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mol/L triethylamine acetate buffer (TEAA; Transgenomic) at a constant flow rate. The reversed-phase gradient was formed by mixing buffer A (0.1 mol/L TEAA, pH 7.0) and buffer B (250 mL/L acetonitrile in 0.1 mol/L TEAA, pH 7.0). Oven temperature for optimum separation of heteroduplex molecules was deduced from 55 °C, 30 s at 72 °C, and 30 s at 94 °C. A final extension of 5 min was carried out at 72 °C on a GeneAmp PCR System 2400 (Perkin-Elmer). DHPLC was carried out on a WAVE DNA fragment analysis system (TransgenomicTM) equipped with a DNASe⁰ column (Transgenomic). DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mol/L triethylamine acetate buffer (TEAA; Transgenomic) at a constant flow rate. The reversed-phase gradient was formed by mixing buffer A (0.1 mol/L TEAA, pH 7.0) and buffer B (250 mL/L acetonitrile in 0.1 mol/L TEAA, pH 7.0). Oven temperature for optimum separation of heteroduplex molecules was deduced from

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence, 5’–3’</th>
<th>Position, nt</th>
<th>Accession no.</th>
</tr>
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<td>NPM Rev3</td>
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<td>1112–1081</td>
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</tr>
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</table>

Eight of the 56 analyzed samples were scored as NPMmut and 48 as NPMwt. The NPM1 mutational status detected in 2 representative cases is shown in Fig. 1. All NPMmut samples and 10 NPMwt samples were also analyzed by reverse transcription-PCR and direct sequencing, which confirmed in all cases the results obtained by DHPLC. The French–American–British subtypes of NPMmut cases were as follows: M0, 2 cases; M1, 1 case; M4, 2 cases, M5a, 1 case; M5b, 2 cases. With respect to cytogenetic characterization, 7 of the NPMmut patients had normal karyotypes, and 1 had deletion of both chromosomes 5q and 7q. As to the type of mutation detected, in 7 NPMmut cases we found the most frequently reported mutation type: a duplication of TCTG tetranucleotide at positions 956–959 of the reference sequence (NM_002520). In the fifth case, nucleotides 965–969 (GGAGG) were substituted by the 9mer GCTT-GAGTC. In all mutated cases, the resulting frameshift led to a product 5 amino acids longer with the new C-terminal tail CFSQVSLRK, peculiar to the NPM1-mutated product. Because NPM1 mutations currently are the most frequently reported genetic aberration in AML and in light of their potential prognostic significance, we believe that inclusion of this DHPLC assay in diagnostic evaluations may improve genetic characterization of AML and allow assignment of patients to better-defined risk categories. Compared with immunohistochemical analysis to detect aberrant cytoplasmic NMP1 localization, this assay overcomes the need to obtain a bone marrow biopsy at the time of AML diagnosis, which is not an established procedure in the routine diagnostic work-up of acute leukemia. Moreover, the DHPLC approach is less time-consuming and less expensive than direct NPM1 sequencing and could therefore represent a suitable technique for rapid screening of AML.

In this study we established a DHPLC-based assay to routinely detect NPM1 mutations in AML and suggest its inclusion in the genetic diagnostic work-up of this disease. With the melting temperature predicted by the Navigator software, we were able to distinguish heteroduplex from homoduplex peaks. The chromatogram from each tested sample was overlaid with the wild-type profile, and samples with an extra peak were scored as mutated: the first peak corresponding to the heteroduplex was detected at 4.3 min, and the second, corresponding to the homoduplex product, was detected at 5 min.

The study recently reported by Falini et al. (5) showed that NPM1, already described to be involved in rearrangements in leukemia (10) and lymphomas (11), is mutated in the leukemic cells of ~35% of primary adult AML. This finding was based on direct sequence analysis of leukemic DNA derived from patients with aberrant dislocation of the NPM1 protein as revealed by immunohistochemistry. In this study we established a DHPLC-based assay to routinely detect NPM1 mutations in AML and suggest its inclusion in the genetic diagnostic work-up of this disease. With the melting temperature predicted by the Navigator software, we were able to distinguish heteroduplex from homoduplex peaks. The chromatogram from each tested sample was overlaid with the wild-type profile, and samples with an extra peak were scored as mutated: the first peak corresponding to the heteroduplex was detected at 4.3 min, and the second, corresponding to the homoduplex product, was detected at 5 min.

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Unusual Rearrangement of the α-Globin Gene Cluster Containing Both the $-\alpha^{3.7}$ and αααanti-4.2 Crossover Junctions: Clinical Diagnostic Implications and Possible Mechanisms, Wen Wang, Amy Y.Y. Chan, Li-Chong Chan, Edmond S.K. Ma, and Samuel S. Chong (Department of Pediatrics, National University of Singapore, Singapore; Division of Hematology, Department of Pathology, The University of Hong Kong and Queen Mary Hospital, Hong Kong, People's Republic of China; Molecular Diagnosis Center, National University Hospital, Singapore; * address correspondence to this author at: Department of Pediatrics, National University of Singapore, Level 4, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074, Singapore; fax 65-6779-7486, e-mail paecs@nus.edu.sg)

Misalignment of the homologous regions of the α-globin gene cluster and unequal crossover during meiosis produce single α-globin gene deletions ($-\alpha$) and reciprocal α-globin gene triplications (ααα). Further unequal crossover of such recombinant alleles with wild-type alleles may produce more complex derivative alleles, such as quadruplicated alleles (1–3). Complex crossover events producing “patchwork” genes have also been reported at the human α- and β-globin gene cluster (4–6). In this report, we describe the identification of a novel rearrangement of the α-globin gene cluster containing both the $-\alpha^{3.7}$ and αααanti-4.2 crossover junctions. This allele was identified in 2 unrelated individuals and a parent in the course of screening by Southern analysis of patients with β-thalassemia major and minor for α-globin gene deletions (Table 1). For patient 1, a routine α-globin gene configuration Southern analysis was performed to screen for the presence of the $-\alpha^{3.7}$ α-thalassemia deletion, a common amelioration factor of severe β-thalassemia (7). In the case of patient 2, Southern analysis was performed to rule out the presence of the $-\alpha^{3.7}$ α-thalassemia deletion, because hemoglobin H inclusion bodies typically present in α-thalassemia are absent when there is concurrent β-thalassemia (8).

Southern analysis was performed by hybridizing [32P]dATP-labeled α- or ω-globin gene probes to BamHI or BglII-digested genomic DNA. With an α-globin probe, an αααanti-4.2 triplication contributes an 18.2-kb hybridizing BamHI band and 16.8- and 7.4-kb BglII bands, whereas a $-\alpha^{3.7}$ deletion contributes a 10.3-kb BamHI band and a 16.3-kb BglII band. With a ω-globin probe, both the αααanti-4.2 and $-\alpha^{3.7}$ alleles contribute 5.9-kb and 10.8/11.3-kb BamHI bands, whereas αααanti-4.2 contributes 11.3/12.6-kb and 16.8-kb BglII bands, and $-\alpha^{3.7}$ contributes 11.3/12.6-kb and 16.3-kb bands.

Southern analysis of the DNA of both patients revealed, instead, an unusual ~20-kb BglII band when hybridized with either the α- or ω-globin probe (Fig. 1A). This anomalous fragment was not consistent with any known deletion or triplication of the α-globin locus and was attributed initially to a polymorphism on one allele that abolished the recognition sequence at an internal BglII site located between the α2- and α1-globin genes.