A novel type of *NPM1* mutation characterized by multiple internal tandem repeats in a case of cytogenetically normal acute myeloid leukemia

The *NPM1* gene, mapped on chromosome 5q35, encodes a nucleolar phosphoprotein composed of 294 amino acids. The NPM1 protein continuously shuttles between the nucleus and the cytoplasm (with predominant nucleolar localization) and is involved in several cellular processes, including centrosome duplication, molecular chaperoning, ribosome biogenesis, DNA repair, and genome stability.[1,2]

Somatic *NPM1* mutations are among the most frequent mutations in acute myeloid leukemia (AML). Overall, *NPM1* mutations occur in approximately one-third of *de novo* AML and more than one half of cytogenetically normal AML in adult patients.[3,4] AML with mutated *NPM1* is recognized as a unique entity according to the 2016 World Health Organization classification of hematologic malignancies.[5] The prognosis of patients with *NPM1*-mutated AML in the absence (or with a low allelic ratio) of *FLT3*-internal tandem duplication (*FLT3*-ITD) is considered as favorable. Patients with this type of AML are not, therefore, retained as candidates for allogeneic stem cell transplantation in first remission in current practice.[6]

To date, more than 50 different mutations located within exon 11 (formerly identified as exon 12) of the *NPM1* gene have been identified. More than 95% of these mutations consist of a net insertion of four base pairs (bp) between nucleotides at position 863 and 864. Three mutation types (A, B, and D) represent about 90% of *NPM1* mutations: the type A mutation (c.860_863dupTCTG) accounts for 70–80% of cases while mutations B and D (c.863_864insCATG and c.863_864insCCTG respectively) together account for 15–20%.[7,8]

Despite their relative heterogeneity, all identified variants are heterozygous and cause reading frame shifts in the region encoding the C-terminal part of the protein. As a consequence, all known *NPM1* exon 11 mutations will removed tryptophan residues at positions 288 and 290 (or 290 alone), which are critical for retaining NPM1 in the nucleolus, and create a leucine-rich nuclear export signal leading to mislocalization of the *NPM1*-mutated protein in the cytosol.[1,2,9] Since the NPM1 mutant protein retains all functional domains in its N-terminal part, it is likely that it is able to interact with several other cytoplasmic proteins contributing to leukemogenesis.[1]

Here, we report a case of AML with a large *NPM1* mutation characterized by multiple internal tandem repeats (*NPM1*-ITR) within exon 11 leading to the creation of a putative elongated protein of 333 amino acids containing a leucine-rich nuclear export signal in its C-terminal part.

The patient was a 73-year old woman referred to our center with a 1-month history of anorexia, a digestive disorder, impaired general condition and weight loss. On

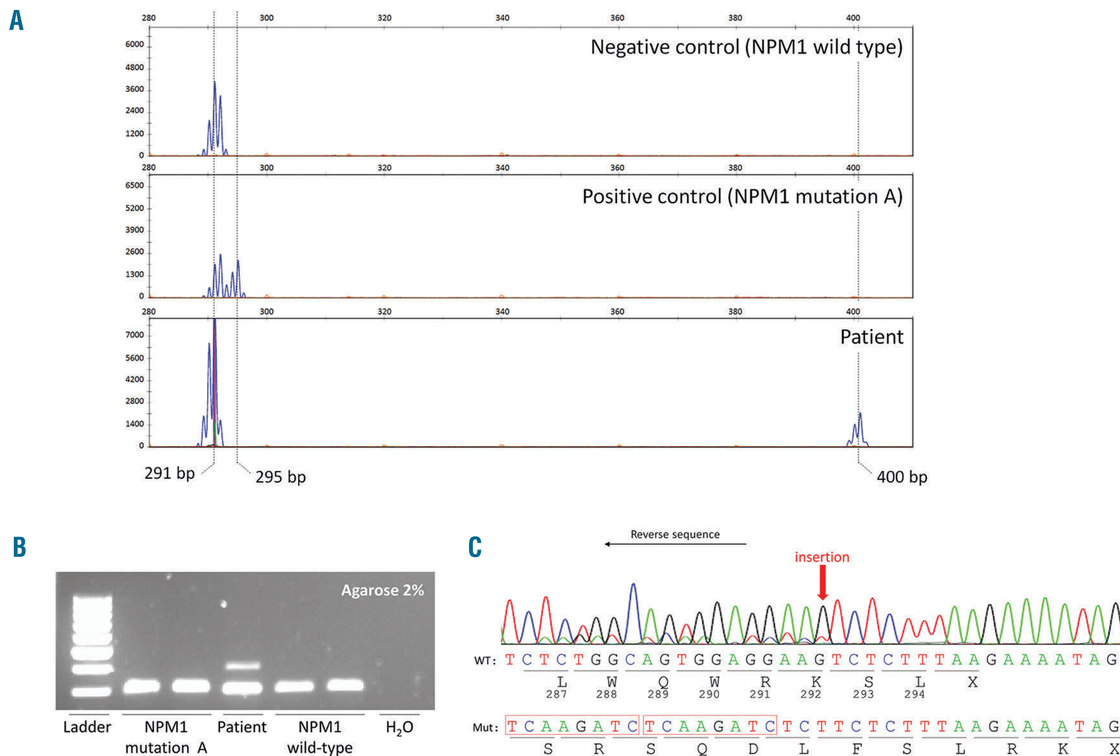

Figure 1. Identification of the *NPM1* mutation. (A) *NPM1* mutation screening by fragment analysis. Size analysis of PCR amplification products can distinguish wild-type *NPM1* (291 bp; negative control; top) from the mutated *NPM1* with a 4 bp insertion (295 bp; positive control; middle) and the novel large insertion (400 bp; present case; bottom). (B) Migration of PCR amplification products on a 2% agarose gel stained with ethidium bromide. An extra band is clearly visible in the patient's sample (about 100 more base pairs than the wild-type product). (C) *NPM1* sequence analysis in the patient (reverse sequence). An arrow indicates the site of insertion. The putative mutated amino acid sequence is shown below.

arrival in our department, the full blood count showed anemia (hemoglobin concentration 8.6 g/dL), thrombocytopenia (platelet count 134 × 10^9/L) and a leukocyte count of 9.3 × 10^9/L including 33% of blast cells, some of which contained Auer rods. A bone marrow aspirate showed 55% of myeloblasts with cytoplasmic azurophilic granules and/or Auer rods and confirmed the diagnosis of AML with maturation (M2 subtype according to the French-American-British classification). Immunophenotyping of blast cells showed coexpression of CD13, CD33, and CD117 markers with a lack of CD34 expression (a feature of AML with mutated *NPM1*). Cytogenetic analysis revealed a normal 46,XX karyotype. Molecular testing was not performed at this time. The woman was given standard induction chemotherapy consisting of anthracycline plus cytarabine (3+7) followed by 6-monthly consolidation courses allowing long-term complete remission.

However, 10 years after the initial diagnosis, at the age of 83 years, she was admitted because of fever associated with neutropenia and circulating blasts. The biological investigations confirmed a relapse of cytogenetically normal AML with 40% bone marrow blasts showing an immunophenotype similar to that previously described. Unfortunately, the patient rapidly died of pulmonary infection. Polymerase chain reaction (PCR) and fragment analysis for *FLT3*-ITD screening gave negative results. Direct Sanger sequencing did not detect mutations in *CEBPA* or the *FLT3*-tyrosine kinase domain (*FLT3*-TKD). PCR and fragment analysis for *NPM1* mutations (GeneScan, 3130 sequence detection system, Applied Biosystems) gave negative results at a first glance. Interestingly, however, when studying the full profile, an additional peak was found in an unexpected region more than 100 bp after the normal wild-type peak (Figure 1A). This finding contrasted with the usual mutated profile associated with all published *NPM1* mutations resulting from a 4-bp insertion[10] (Figure 1A). Migration of PCR amplification products according to their size on a 2% agarose gel identified an extra band in the patient's sample (Figure 1B). Finally, direct Sanger sequencing (ABI PRISM 3130XL genetic analyzer, Applied Biosystems) clearly showed a large insertion in the *NPM1* gene. Sequence analysis revealed the deletion of nucleotides 868 to 876 followed by the insertion of 118 nucleotides (c.868_876delins118:p.W290Ifs*45) (Figure 1C). The large insertion consisted of 13 tandem repeats of nucleotides 852 to 859 (TCAAGATC) or 852 to 862 (TCAAGATCTC). The predicted mutated protein was 333 bp in size (compared to 294 bp for the wild-type protein and 298 bp for type A, B or D mutated proteins). The tryptophan residue 290 was lost whereas the tryptophan residue 288 was retained. As reported for mutations A, B and D, this mutation is expected to create a new nuclear export signal leading to *NPM1* mislocalization in the cytosol according to the LocNES (Locating Nuclear Export Signals) prediction tool[11] (Figure 2 and *Online Supplementary Table S1*).

Extensive mutational analysis by high-throughput sequencing (Ampliseq panel, Proton sequencing, Life Technologies) identified concomitant mutations in *CBL* [D460dup; variant allele frequency (VAF) 28%], *DNMT3A* (D531N; VAF 31%), *IDH1* (R132H; VAF 28%), *NRAS* (Y64N; VAF 4%), and *TET2* (W1847X; VAF 38%) all of which are frequently encountered in *NPM1*-mutated cases of AML.[3] VAF of the *NPM1*-ITR mutation could not be interpreted because of important ambiguities in alignment and assembly due to tandem repetitions. Retrospective analysis of the diagnostic sample (10 years previously) found the same mutations in *CBL* (VAF 50%), *DNMT3A* (VAF 3%) and *TET2* (VAF 47%). However, the *NPM1*-ITR mutation was absent (using high-throughput sequencing and fragment analysis) showing that it had been acquired at the time of relapse.

NPM1 mutations are one of the most frequent mutations and the most important markers in AML. Importantly, the fragment analysis method is one of the most commonly used for detection of *NPM1* mutations.

Figure 2. Nucleotide and amino acid sequences of the novel *NPM1* mutation identified in this report (NPM1-ITR). The large insertion consists of 13 repetitions of the nucleotides TCAAGATCT(CT). Tryptophan 290 of the NoLS in the wild-type NPM1 (gray background) is replaced by another amino acid whereas tryptophan 288 is retained. Mutations A, B and D are also represented. The putative leucine-rich NES motif found in mutated NPM1 proteins is shown (underlined). NES: nuclear export signal; NLS: nuclear localization signal; NoLS: nucleolar localization signal.

Because these mutations almost always involve a 4-bp insertion, fragment analysis should be interpreted with caution in order not to misdiagnose a rare, larger insertion. Functional interactions with other proteins as well as the stability of the NPM1-ITR mutant protein is difficult to evaluate and could be different from those of classical NPM1 mutant proteins. However, the patient reported here had the main biological features of AML with mutated *NPM1* (normal karyotype, CD34 negativity, common concurrent mutations).[3,12] Moreover, the *NPM1*-ITR mutation is supposed to remove the tryptophan 290 residue and create a leucine-rich nuclear export signal, a feature shared by all NPM1 mutant proteins reported in the literature.[7-9] In conclusion, we describe, to our knowledge, the first report of an *NPM1*-ITR mutation in AML with potential implications for its detection in routine practice.

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