NPM1 mutation subtype switch in acute myeloid leukemia

Mutations in exon 11 of the Nucleophosmin (*NPM1*) gene are detected in approximately 40% of adult acute myeloid leukemias (AML) with a normal karyotype.[1] Since their discovery, more than 70 different *NPM1* mutations have been described in exon 11.[2] The most common mutation, representing about 70% of *NPM1* mutations, is a TCTG tetranucleotide duplication (type A). Other common mutations, accounting for 10% and 5% of cases respectively, involve the insertion of CATG (type B) or CCTG (type D) at the same nucleotide position. Rare mutations or translocations involving exons 5, 9, and 10 have also been observed in AML.[3]

NPM1 mutations were first included as a specific AML subtype-defining abnormality in the 4th classification of the World Health Organization (WHO) in 2008, with the blast cut-off being finally lowered in more recent classifications (both WHO and International Consensus Classification).[4,5] Numerous studies have established the favorable prognosis of *NPM1*-mutated AML, especially in the absence of *FLT3*-internal tandem duplication (ITD).[6]

Studies of paired diagnosis and relapse samples have shown that *NPM1* mutations remain highly stable throughout the disease, making them an effective marker for measurable residual disease (MRD) monitoring.[7,8]

Here, we present 3 cases of *NPM1*-mutated AML who developed a second leukemic episode with a different *NPM1* mutation, accompanied by phenotypic changes. All patients were initially treated with intensive chemotherapy alone without allogeneic hematopoietic stem cell transplantation (ASCT) in first complete remission or targeted therapy or immunotherapy and none received maintenance therapy before the disease recurrence. In all patients, mutation change was identified by high-throughput sequencing (HTS) and subsequently quantified using reverse transcription (RT)-quantitative PCR (qPCR) for type A/B/D variants or RT-digital droplet PCR (ddPCR) for other *NPM1* variants. *IDH2* mutations were retrospectively monitored using genomic DNA-based ddPCR (threshold 0.1% variant allele frequency [VAF]) in all patients. Additionally, a single-cell proteogenomic analysis was conducted in one patient. This study was approved by an Institutional Review Board and conducted in accordance with the Declaration of Helsinki.

The first patient (UPN1) was a 29-year-old adult with AML. Immunophenotypic analysis of bone marrow (BM) at diagnosis revealed 56% of CD34-negative blasts with a myeloid phenotype (CD33^{+}). Cytogenetic analysis showed a normal karyotype. HTS identified mutations in *NPM1* (type A variant), *IDH2*, *FLT3*-ITD (detected by fragment analysis) and *KRAS* (Table 1, Figure 1A). The patient received intensive chemotherapy and achieved undetectable BM-*NPM1* MRD by RT-qPCR at the end of treatment. At this point, *IDH2* mutation was also undetectable by ddPCR. Two years after diagnosis, the patient experienced a second leukemic episode with the same *IDH2*-mutated founding clone but displayed an *NPM1* mutation switch to a new mutation (i.e., c.863_864insTCGG) along with a new *FLT3*-TKD1 subclone. MRD monitoring of the type A variant by RT-qPCR remained negative. Additionally, immunophenotyping showed the loss of CD33 expression and aberrant expression of CD56 compared to the first leukemic episode. The disease recurrence was treated with azacytidine and venetoclax, followed by ASCT in complete remission. To date the patient is still in remission.

The second patient (UPN2) was a 60-year-old adult with AML. Morphological examination of BM at diagnosis revealed infiltration of 82% of myeloid blasts. Karyotype was normal and HTS revealed mutations in *IDH2*, *NPM1* (type A variant), *SRSF2*, *RUNX1* and *FLT3*-JMD. The patient received intensive chemotherapy resulting in undetectable BM-*NPM1* MRD by RT-qPCR while the *IDH2* mutation was still detectable by ddPCR with a VAF of 5.27%. A second leukemic episode occurred five years after diagnosis and molecular analysis identified the same *IDH2* and *SRSF2* mutations with an *NPM1* mutation switch (i.e., 863_864insCTCG) along with the acquisition of new mutations in *ASXL1* and *FLT3*-TKD1/2 (Figure 1B). The patient underwent an ASCT, allowing a durable complete remission.

The third patient (UPN3) was a 57-year-old man. Morphological examination of BM at diagnosis showed infiltration of 87% of myeloid blasts. Karyotype was normal and HTS revealed mutations in *IDH2*, *SRSF2* and *NPM1* (type A variant). The patient received intensive chemotherapy resulting in undetectable BM-*NPM1*-MRD by RT-qPCR while the *IDH2* was still detectable by ddPCR with a VAF of 11.2%. A new leukemic episode occurred four years after initial diagnosis. At this time, blasts showed a monoblastic phenotype: CD117^{-}, CD11b^{+}, HLA-DR^{+}, CD36^{+}, CD4^{+}. The *IDH2* and *SRSF2* mutations were still present, along with a *NPM1* mutation switch (i.e., c.863_864insTCAG) and emerging mutations in *ASXL1*, *KRAS* and *SMC1A* (Figure 1C). *NPM1* mutation type A was quantified at 1.31% by RT-qPCR while the newly acquired *NPM1* mutation (i.e., c.863_864insTCAG) was quantified by RT-ddPCR at 113%. We were unable to determine whether the low type A mutation signal was due to cross-reactivity with the new *NPM1* variant or to persistence of the initial mutation at very low levels (the type A variant was not detected in the HTS raw data). The patient died after two weeks, following a rapid deterioration of his general condition.

To explore the clonal hierarchy in greater depth, we conducted a single-cell analysis on cryopreserved cells of UPN1, at both leukemic episodes. Due to low cell viability at diagnosis, surface marker analysis could not be performed. The single cell genomic and proteogenomic assays were carried out on the Tapestri platform (MissionBio®, USA). This enabled us to analyze 3,991 cells and 1,814 cells at each episode, respectively. The study of genetic phylogeny

Table 1. Mutational analysis by high throughput sequencing performed at diagnosis and disease recurrence in the 3 patients.

Patient ID	Stage	High throughput sequencing mutational analysis
UPN1	D	*IDH2* exon 4 c.419G>A : p.R140Q (VAF 43%) *NPM1* exon 11 c.860_863dup : p.W288Cfs*12 (VAF 39%) *KRAS* exon 2 c.64C>A : p.Q22K (VAF 33%) *FLT3*-ITD 78 bp (VAF 6%)
	DR	*IDH2* exon 4 c.419G>A : p.R140Q (VAF 41%) *NPM1* exon 11 c.863_864insTCGG : p.W288Cfs*12 (VAF 39%) *FLT3* exon 16 c.2039C>T : p.A680V (VAF 4%)
UPN2	D	*SRSF2* exon 1 c.284C>T : p.P95L (VAF 45%) *IDH2* exon 4 c.419G>A : p.R140Q (VAF 40%) *NPM1* exon 11 c.860_863dup : p.W288Cfs*12 (VAF 40%) *RUNX1* exon 9 c.1430G>A : p.W477X (VAF 1%) *FLT3* exon 13 c.1694A>T : p.K565M (VAF 13%) *FLT3* exon 14 c.1717G>A : p.E573K (VAF 1%) *FLT3* exon 14 c.1727T>G : p.L576R (VAF 1%)
	DR	*SRSF2* exon 1 c.284C>T : p.P95L (VAF 45%) *IDH2* exon 4 c.419G>A : p.R140Q (VAF 40%) *NPM1* exon 11 c.863_864insCTCG : p.W288Cfs*12 (VAF 40%) *ASXL1* exon 12 c.2021_2035delinsT : p.H674Lfs*39 (VAF 2%) *FLT3* exon 16 c.2039C>T : p.A680V (VAF 25%) *FLT3* exon 20 c.2508_2510del : p.I836del (VAF 11%)
UPN3	D	*SRSF2* exon 1 c.284C>A : p.P95H (VAF 51%) *IDH2* exon 4 c.419G>A : p.R140Q (VAF 51%) *NPM1* exon 11 c.860_863dup: p.W288Cfs*12 (VAF 40%)
	DR	*SRSF2* exon 1 c.284C>A : p.P95H (VAF 48%) *IDH2* exon 4 c.419G>A : p.R140Q (VAF 46%) *NPM1* exon 11 c.863_864insTCAG : p.W288Cfs*12 (VAF 2%) *ASXL1* exon 12 c.2035G>T : p.G679X (VAF 4%) *KRAS* exon 3 c.190T>A : p.Y64N (VAF 1.2%) *SMC1A* exon 13 c.2140T>A : p.Y714N (VAF 10%)

D: diagnosis; DR: disease recurrence; ITD: internal tandem duplication; VAF: variant allele frequency.

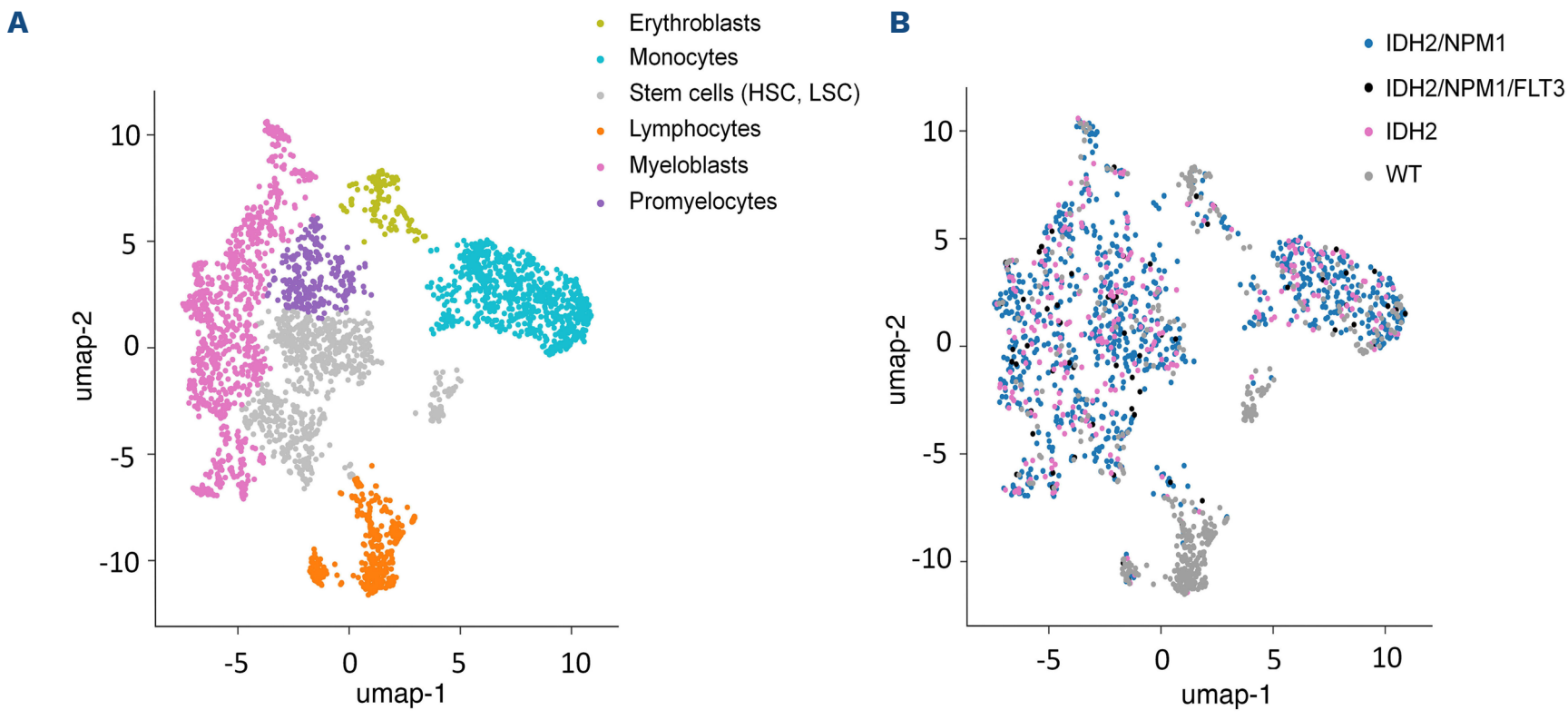

Figure 1. Clonal evolution of *NPM1*-switch cases. (A) Clonal evolution in Patient 1 (UPN1) determined by single-cell analysis. (B and C) Clonal evolution inferred from bulk high throughput sequencing analysis in Patients 2 (UPN2) and 3 (UPN3.) (D) *NPM1* exon 11 sequence at diagnosis and disease recurrence. HSC: hematopoietic stem cells; LSC: leukemic stem cells; WT: wild-type.

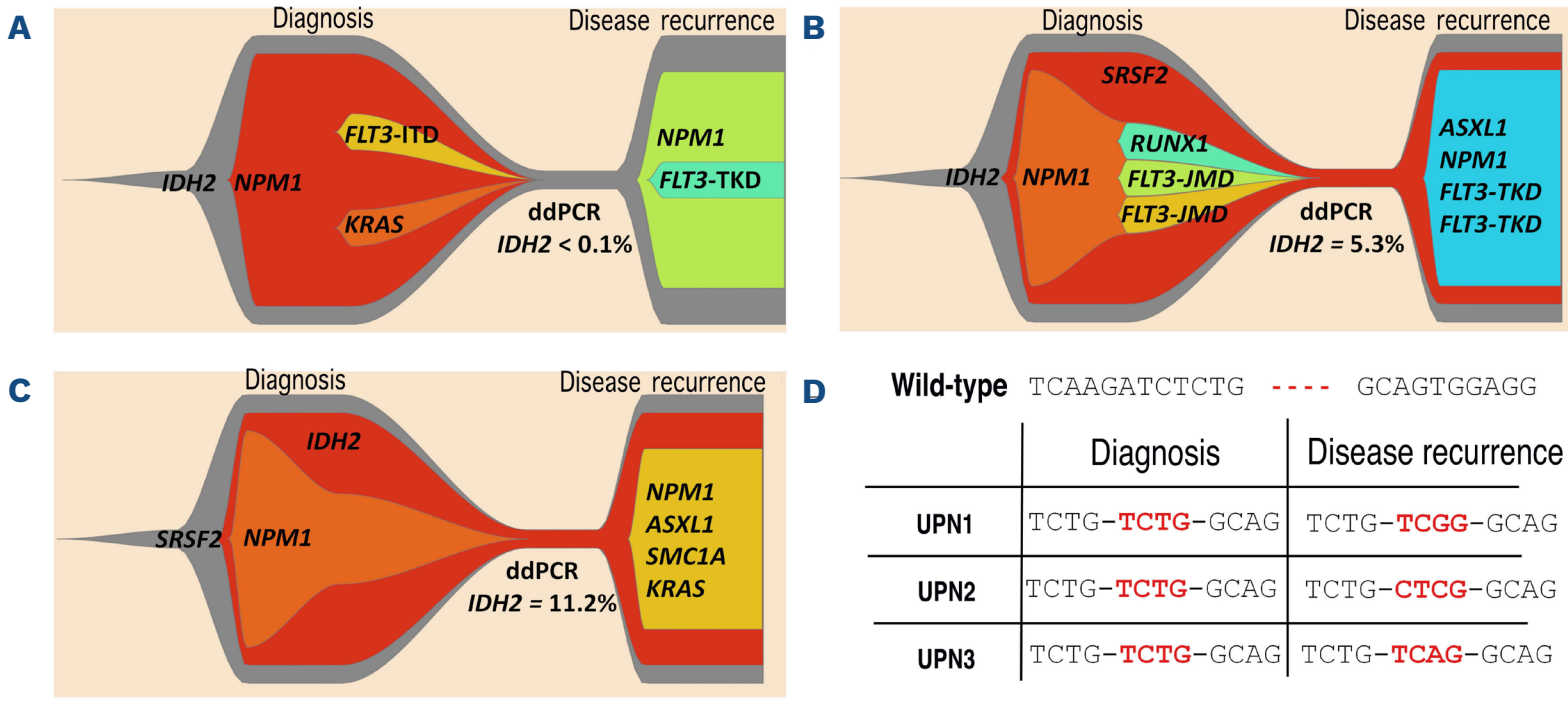

Figure 2. Single-cell analysis at disease recurrence in Patient 1. (A) Single cell proteomic analysis and definition of cell populations in Patient 1 (UPN1). (B) Single cell proteogenomic analysis. HSC: hematopoietic stem cells; LSC: leukemic stem cells; WT: wild-type.

at initial diagnosis by single-cell sequencing confirmed the results inferred from HTS bulk sequencing (Figure 1), except from the *FLT3*-ITD mutation which was undetectable by single-cell sequencing due to bioinformatics issues. The study of the second episode showed that none of the 1,814 analyzed cells carried the *NPM1* type A mutation, and, similarly, the *NPM1* mutation associated with disease recurrence was absent in the 3,991 cells analyzed at initial diagnosis. The proteogenomic analysis revealed that the *NPM1* mutation was present both in the granulo-monocytic ($CD33^+$ $CD117^+$) and erythroblastic ($CD36^+$ $CD71^+$) compartments. Moreover, single cell analyses enabled us to define the stem cell compartment ($CD34^+$ $CD38^-$) as being composed of hematopoietic stem cells (HSC, wild-type) and leukemic stem cells (LSC, mutated) (Figure 2A, B).

To our knowledge, 2 other AML cases with an *NPM1* mutation subtype switch have been reported in the literature.[9,10] Interestingly, in line with our 3 observations, these 2 cases displayed immunophenotypic changes between the 2 episodes. Both patients experienced disease recurrence eight and six years following diagnosis, respectively.[9,10]

NPM1-mutated AML is one of the most common AML entities in adults and it carries a generally favorable prognosis. However, these diseases may relapse, in which case they mostly involve a stable *NPM1*-mutated clone, allowing MRD monitoring through sensitive techniques like RT-qPCR or RT-ddPCR. Here, we present 3 cases demonstrating that *NPM1* mutation switches can occur. All 3 cases harbored an *IDH2* (R140Q) founder clone, raising questions about the promoting effect of such mutation (or subsequent epigenetic changes) in the emergence of *NPM1*-mutated clones. This also raises the question of whether this new leukemic episode should be considered as a relapse or as the emergence of a second '*novo*-like' leukemia (in line with the delayed onset of the second episode, suggesting a long period of underlying *IDH2*-mutated clonal hematopoiesis), which could confer a chemosensitivity profile and prognosis distinct from true relapses.

Together with cases from the literature, these observations illustrate pitfalls in MRD monitoring. While *NPM1*-specific PCR assays are recommended by international guidelines, these observations argue for extensive genetic screening at the time of relapse (also allowing the detection of new actionable targets) and the combination of other methods or markers (i.e., flow cytometry, *WT1* expression). The use of single cell genomics and HTS-based MRD approaches to track both the founder clone and the emergence of new mutations (including new *NPM1* mutations) is a current topic of interest.[11,12] This is all the more relevant in the context of developing targeted therapies, such as IDH inhibitors or menin inhibitors, which have shown activity against *NPM1*-mutated clones.

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Contributions
BP, RJ, CP and ND collected data and drafted the manuscript. BP, RJ, CR and MB performed single cell analysis. BP and AB analyzed single cell analysis. FD and CR performed flow cytometry analysis. ND, MB, BP, MD and CP acquired and analyzed molecular data. CB, LF and LG provided samples and clinical data. All authors critically revised the manuscript and approved the final version for publication.

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Data-sharing statement
Data are available upon reasonable request to the corresponding author.

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