PU.1 subcellular localization in acute myeloid leukaemia with mutated *NPM1*

NPM1 is one of the most frequently mutated genes in acute myeloid leukaemia (AML), with one third of AML patients carrying *NPM1* mutations (Falini *et al.*, 2005). NPM1 is a multifunctional nucleolar chaperone, involved in key biological processes such as maintenance of genome stability and ribosome biogenesis (Brunetti *et al.*, 2019). *NPM1* mutations are typically heterozygous four base-pair insertions in the last exon of the gene (Falini *et al.*, 2007) that result in the generation of a novel C-terminal nuclear export signal (Bolli *et al.*, 2007). Therefore, in contrast with the nuclear localization of the wild-type protein, mutant NPM1 (NPM1c) localizes to the cytoplasm of leukaemic cells.

Data from patients, cell lines and murine models indicate that *NPM1* mutations are AML drivers (Heath *et al.*, 2017). Although NPM1c is necessary for AML maintenance (Brunetti *et al.*, 2018), the mechanisms through which it promotes and maintains leukaemia are still unclear. One hypothesis is that NPM1c would relocate nuclear proteins involved in myeloid differentiation (e.g. transcription factors) to the cytoplasm, blocking their normal function. In agreement, a recent study has shown that NPM1 interacts with the myeloid transcription factor PU.1, relocating it to the cytoplasm of *NPM1*-mutated AML models (Gu *et al.*, 2018). Based on these observations, we sought to confirm the cytoplasmic localization of PU.1 in primary *NPM1*-mutated AML samples and to determine whether PU.1 localization, studied by immunohistochemistry (IHC) in bone marrow biopsies, could be used as a surrogate for predicting *NPM1* mutational status. To expand our analysis, we also explored PU.1 localization in two *NPM1* wild-type and two *NPM1*-mutated AML cell lines through western blotting (WB) of nuclear and cytoplasmic fractions and immunofluorescence (IF).

The study was approved by the Local Institutional Board and all patients signed a written informed consent prior to the bone marrow biopsy. For IHC studies, bones were fixed in B5 solution, decalcified in ethylenediaminetetraacetic acid and embedded into paraffin. Antigens were unmasked by incubating paraffin sections in EnVision FLEX Target Retrieval Solution High pH (Dako-Agilent, Santa Clara, CA, USA). Cell lines were cultured in complete RPMI 1640 and MEM Alpha media. For subcellular fractionation, we used the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) starting from 5×10^6 cells and following the manufacturer's instructions. Whole cell lysates as well as nuclear and cytoplasmic fractions were run on 4–15% precast gels and transferred onto polyvinylidene fluoride membranes. Two gels were loaded simultaneously for parallel blotting. For IF, 5×10^4 cells were cytospinned onto slides, fixed with 4% paraformaldehyde (PFA), permeabilized with 0·1% Triton X-100 and blocked with 1% bovine serum albumin. Primary antibodies, dilutions and incubation times used for each experiment are summarized in Table SI.

To determine PU.1 localization in primary AML cells, we performed IHC on sections from 39 bone marrow biopsies characterized by a high blast count, either at diagnosis or at relapse. We studied 17 *NPM1* wild-type and 22 *NPM1*-mutated samples using a commercially available PU.1 antibody (referred to as ab1), together with an antibody directed against the N-terminus of NPM1 (recognizing both wild-type and mutant forms, referred to as tNPM1). Surprisingly, PU.1 was localized to the nucleus of all *NPM1*-mutated samples (Fig 1A and Figure S1), with only 4 of 22 cases displaying weak cytoplasmic staining. Similarly, PU.1 localization was nuclear in all *NPM1* wild-type samples (Fig 1A and Figure S1), with 3 of 17 cases also showing weak cytoplasmic staining. As expected, IHC with tNPM1 confirmed nuclear NPM1 localization in all *NPM1* wild-type samples and simultaneous nuclear and cytoplasmic localization of NPM1 in all *NPM1*-mutated cases (Fig 1A and Figure S1). To confirm these results, we repeated IHC on 12 of 39 biopsies (six *NPM1* wild-type and six *NPM1*-mutated) using a different commercially available PU.1 antibody (referred to as ab2). Again, PU.1 localized to the nucleus in all cases (Fig 1A and Figure S1) with only faint cytoplasmic staining in two *NPM1* wild-type and one *NPM1*-mutated sample (Figure S1 – patients 5, 6 and 8 respectively). In summary, IHC of primary bone marrow biopsies did not reveal clear differences in PU.1 localization between *NPM1* wild-type and *NPM1*-mutated samples and was not able to demonstrate cytoplasmic localization of PU.1 in mutated cases.

To validate our observations, we analyzed PU.1 localization in two *NPM1*-mutated (OCI-AML3 and IMS-M2) and two *NPM1* wild-type (OCI-AML2 and HNT34) AML cell lines. IF with either ab1 or ab2 revealed strong staining for PU.1 in the nuclei, but no cytoplasmic localization in any cell line (Fig 1B and Figure S2). Staining with tNPM1 confirmed simultaneous nuclear and cytoplasmic localization of NPM1 in *NPM1*-mutated cells and nuclear staining in *NPM1* wild-type cells (Fig 1B). To validate IF results, we evaluated PU.1 expression in whole-cell lysates from AML cells (Figure S3) and then performed nuclear and cytoplasmic fractionation of the same cell lines, followed by WB

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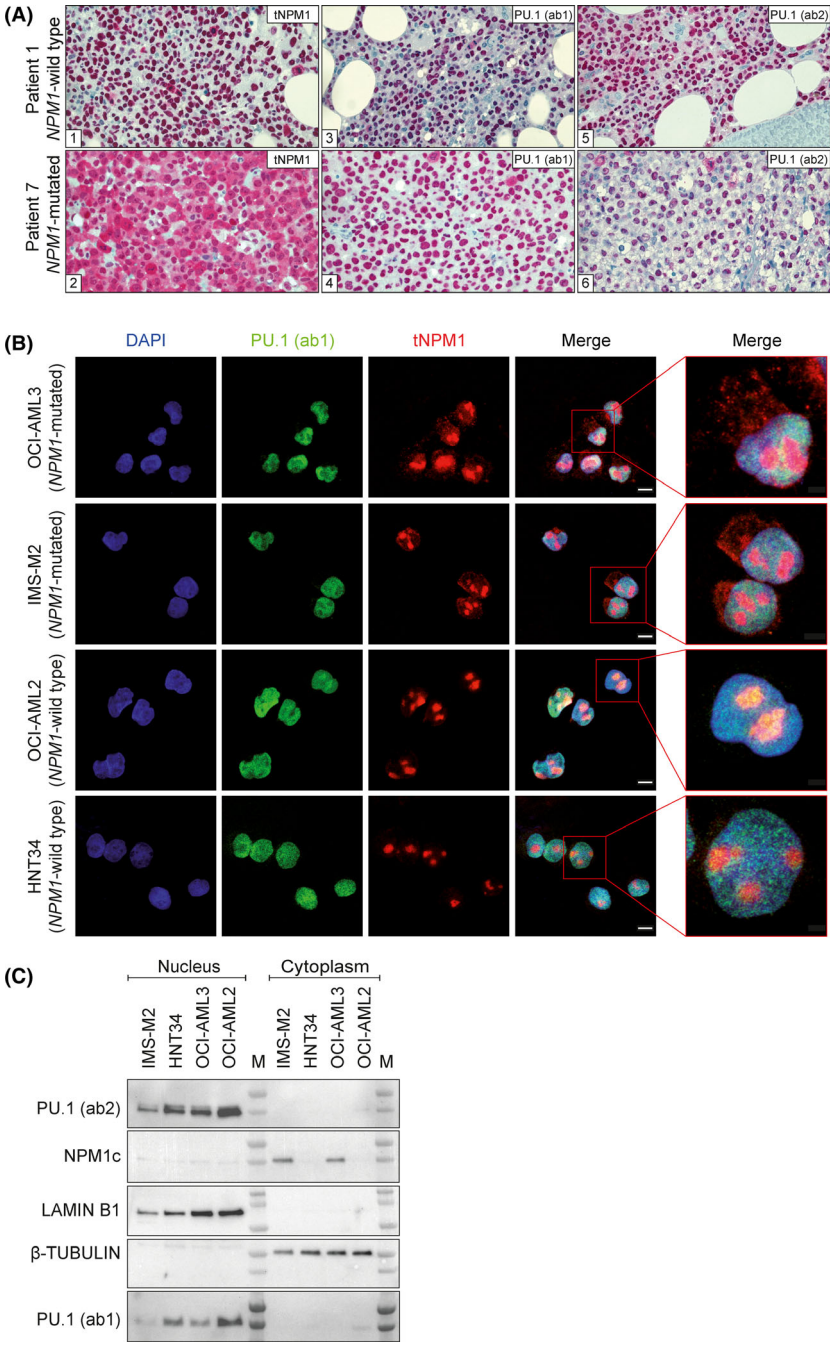

Fig 1. PU.1 subcellular localization in bone marrow biopsies from AML patients and in AML cell lines. (A) Immunohistochemistry of bone marrow biopsies from AML patients. tNPM1, PU.1 ab1 and PU.1 ab2 staining of one representative *NPM1* wild-type (Patient 1, quadrants 1, 3 and 5) and one representative *NPM1*-mutated (Patient 7, quadrants 2, 4 and 6) AML sample. Images were taken at 40× magnification with an Olympus BX51 microscope equipped with an Olympus DP71 digital camera. (B) Immunofluorescence with tNPM1 (red) and PU.1 ab1 (green) antibodies in OCI-AML3 and IMS-M2 cells (*NPM1*-mutated) and OCI-AML2 and HNT34 cells (*NPM1* wild-type). Nuclei were stained with DAPI (blue). Images were collected with the Zeiss LSM800 confocal microscope equipped with a 63× immersion objective. Scale bars: 10 μm. An enlarged view of the cells framed in red is shown on the right. (C) Western blot analysis of nuclear and cytoplasmic fractions obtained from *NPM1* wild-type (HNT34 and OCI-AML2) and *NPM1*-mutated (IMS-M2 and OCI-AML3) AML cell lines. Blots were probed with anti-lamin B1 and anti-β-tubulin antibodies to confirm the purity of nuclear and cytoplasmic fractions respectively. Anti-NPM1c antibody was used to confirm the presence of mutant NPM1 in the cytoplasmic fractions of *NPM1*-mutated cells. Blots were probed with both ab1 and ab2 anti-PU.1 antibodies to evaluate the PU.1 subcellular localization. M, molecular weight marker.

analysis. Immunoblotting with either ab1 or ab2 confirmed nuclear localization of PU.1, with no clear signal in any of the cytoplasmic fractions (Fig 1C). Staining with an anti-NPM1c antibody confirmed the presence of mutant NPM1 in the cytoplasmic fractions of *NPM1*-mutated cells (Fig 1C). Altogether, IF and WB data in AML cell lines corroborate our observation in primary AML samples, indicating that PU.1 localization is not altered by the presence of NPM1c in leukaemic cells.

AML with mutated *NPM1* is one of the most frequent subtypes of leukaemia in adults. About 50% of adult patients diagnosed with *NPM1*-mutated AML eventually die of leukaemia and the prognosis is even worse in elderly patients (Brunetti *et al.*, 2019). A better understanding of the molecular mechanisms downstream of NPM1c is necessary to open new therapeutic avenues for this frequent AML subtype. Here, in contrast with previous results, we failed to show cytoplasmic PU.1 localization in primary AML samples and AML cell lines with mutated *NPM1*. The discrepancy between previously published data and our results may be partially explained by some technical differences. First, our work is founded on IHC in primary patient samples, while previous studies were focused on AML models only. Second, while previously published work used methanol-based fixation (Gu *et al.*, 2018), in our study IF was performed on PFA-fixed cells, which may have limited the detection of

PU.1 in the cytoplasm. However, the overlap between IHC results in primary samples and IF and WB results in cell lines supports our conclusions. Although we cannot exclude that sporadic AML cases may show partial cytoplasmic localization of PU.1, we conclude that subcellular PU.1 localization should not be used to predict *NPM1* mutations in AML patients. Further studies are needed to clarify the proportion and the biological relevance of PU.1 delocalization in AML with mutated *NPM1*.

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Author contributions

GP and CB designed the experiments. GP performed the experiments and analyzed the data. BB collected patient samples and performed IHC. RR acquired IHC and IF pictures. LB supervised the study and wrote the letter with GP and CB. All authors approved the manuscript.

Competing interests

The authors declare no competing interests.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. *NPM1* and PU.1 immunohistochemical staining in patients' bone marrow biopsies.

Fig S2. *NPM1* and PU.1 immunofluorescence in AML cell lines.

Fig S3. Western blot of whole cell lysates of AML cell lines.

Table SI. Primary antibodies used in the experiments.

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