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A novel mechanism of NPM1 cytoplasmic localization in acute myeloid leukemia: The recurrent gene fusion NPM1–HAUS1

Running heads: NPM1-HAUS1 causing cytoplasmic NPM1 localization in AML


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PVC designed the study, analyzed the data, planned the experiments, wrote and reviewed the manuscript.
FPSS designed the study, analyzed the data, planned the experiments and reviewed the manuscript.
WOP, BL and LM performed the experiments, analyzed the data and reviewed the manuscript.
RP performed bioinformatics analysis and reviewed the manuscript.
ERP performed cytogenetic analysis and reviewed the manuscript.
AMPSB made the morphological analysis and reviewed the manuscript.
JCCG, FFC and NH analyzed the data and reviewed the manuscript.
KNM analyzed the data, performed experiments and reviewed the manuscript.
RCP performed the experiments, analyzed the data and reviewed the manuscript.

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NPM1 heterozygous mutations are present in roughly a third of patients with acute myeloid leukemia (AML), making it one of the most frequent genomic alterations in these patients.\textsuperscript{1} The mutations are characterized by frameshift insertions in the region encoding the C-terminus of the protein, leading to disruption of tryptophan residues 288 and 290 and generation of an additional nuclear export signal (NES) motif, that ultimately leads to the cytoplasmic localization of the mutated NPM1 (NPM1\textsubscript{m}) as well as wild type (WT) NPM1 proteins.\textsuperscript{2}

The observation that patients with NPM1\textsubscript{m} AML share clinical, prognostic and biological features\textsuperscript{1,3} as well as evidence suggesting NPM1 mutation as a primary and specific event in AML\textsuperscript{3,4} has led to the creation of the provisional entity: “AML with mutated NPM1”, in the 2008 World Health Organization Classification of Tumours of Haematopoietic and Lymphoid tissues.\textsuperscript{5}

It has been reported that a subset of patients with NPM1 cytoplasmic localization do not have detectable NPM1 mutations.\textsuperscript{6} While a fraction of these patients harbor the t(3;5)(q25;q35) (NPM1-MLF1),\textsuperscript{6,7} the remaining patients have, so far, unknown operating genomic mechanisms. The identification of such patients and mechanisms is important since this group could clinically and biologically overlap with the entity “AML with mutated NPM1”. Herein we describe a novel recurrent fusion gene NPM1-HAUS1 identified in two AML patients. This gene fusion leads to cytoplasmic localization of the NPM1 chimeric protein in \textit{in vitro} assays.

Bone marrow and skin biopsy samples were obtained after signing the informed consent of the institutional Review Board (IRB) approved protocol 08942912.0.1001.0071. Bone marrow mononuclear cells (BMMC) were
obtained with the use of Ficoll-Paque (Sigma Aldrich) and whole DNA extraction was achieved with QIAamp DNA mini kit (Qiagen). Sequencing libraries were prepared using the Nextera preparation kit (Illumina) and sequencing with 100 bp paired-end reads was performed on an Illumina HiSeq2000. Somatic variants calls were generated by combining the output of Somatic Sniper (Washington University), Mutect (Broad Institute) and Pindel (Washington University) plus additional in-house criteria to reduce false-positive calls. Median coverage of leukemia and skin sample was 70x and 30x respectively. The search for fusion sequences was performed with the software Factera v1.4.3b.8 Lentiviral vector-based clones fused to fluorophores for 293T cells transduction were manufactured by Genecopoeia. Sanger sequencing was used to confirm the fusion sequence in both patients.

Initially we studied a 63 year old woman (P1) with a diagnosis of de novo AML. Bone marrow examination showed marked hypercellularity with 95% of blasts characterized by medium size, intermediate nuclear:cytoplasmic ratio, presence of nucleolus and basophilic cytoplasm. See Table 1 for clinical and laboratorial characteristics.

Karyotype analysis was consistent with 46,XX,t(5;18)(q35;q21)[20]. Polymerase chain reaction (PCR) fragment analysis revealed absence of exon 12 NPM1 insertions and presence of FLT3 internal tandem duplication (FLT3 ITD). Since t(5;18)(q35;q21) is recurrent in AML9,10 and the genes involved in the translocation have not been identified, with the aim of molecularly characterizing this fusion we performed paired whole genome sequencing of a skin sample and BMMC from the patient. Three oncogenic driver abnormalities were identified by our pipeline: internal tandem duplication (ITD) of the FLT3
gene, a missense DNMT3A mutation (p.S714C) and fusion sequences between chromosomes 5 and 18. The consensus chimeric sequence fused NPM1 intron 11 (NM_002520.6) to HAUS1 intron 8 (NM_NM_138443.3) (Figure 1A). We first demonstrated the expression of the in frame fusion transcript by means of RT PCR and Sanger sequencing (Figure 1B). The putative chimeric protein (Figure 1C) generated by this fusion was very similar to mutated NPM1, in its identical size (298 amino acids), in the disruption of Tryptophan 288 and 290 and the generation of a slightly different NES motif: L-xxx-V-xx-M-x-L instead of L-xxx-V-xx-V-x-L (Figure 1D). We used LocNES11, a computational tool that locates classical NES in proteins and the motif LTRRVDMMEL, corresponding to the C’ terminal region of NPM1_HAUS1 was predicted to be a classical NES with a high probability (score of 0.44. A score above 0.1 is considered significant). In order to evaluate if the novel NES was functional, we transduced 293T cells with lentiviral vectors containing: NPM1_WT-mCherry (wild type NPM1); NPM1m-GFP (NPM1 type A mutation), NPM1_HAUS1-GFP (NPM1-HAUS1) and empty vector-GFP.

While NPM1 WT localized exclusively in the nucleus, both NPM1m and NPM1-HAUS1 displayed the same pattern of nuclear and cytoplasmic localization (Figure 2A). Given that the cytoplasmic localization of NPM1m is mediated by exportin-1,12 we used leptomycin-B, an exportin-1 inhibitor to evaluate the impact of exportin-1 mediated transport in the subcellular localization of both proteins, as previously described.12 Treatment with leptomycin-B abrogated the migration of both proteins to cytoplasm (Figure 2B), suggesting that NPM1-HAUS1 cytoplasmic localization occurs by the same
mechanism of NPM1m localization, the disruption of tryptophan 288 and 290 and the generation of a novel NES signal.

To evaluate if NPM1-HAUS1 gene fusion is recurrent in patients with AML and t(5;18)(q35;q21), we analyzed genomic DNA from a second patient (P2) harboring such translocation, that has been subject of a previous publication \(^9\) (See table 1 for further clinical and laboratorial features). PCR amplification using primers complementary to NPM1 exon 11 and HAUS1 exon 9 followed by Sanger sequencing revealed a similar fusion sequence, with breakpoints that although not identical, occurred in the same introns of both genes (NPM1 intron 11 and HAUS1 intron 8), therefore generating an identical putative protein, confirming the recurrence of the lesion.

AML with mutated NPM1 is the most common form of AML. It is characterized by preponderance of CD34 negative blasts, commonly with monocytic differentiation, and a high correlation with normal cytogenetics, FLT3 and DNMT3A mutations. In addition, several patients with NPM1m AML have a more chemosensitive disease, with long term outcomes similar to patients with other favorable risk AML.\(^{13}\) This fact led to the inclusion of this AML subtype in the favorable risk LeukemiaNet prognostic category (in the absence of poor prognostic FLT3-ITD mutations).\(^{14}\)

It is known that a subset of patients with cytoplasmic NPM1 do not harbor NPM1 mutations,\(^7\) and it has been shown that patients carrying the t(3;5)(q25;q35) and the fusion NPM1-MLF1 are in this group. Nevertheless, the mechanism by which the fusion NPM1-MLF1 causes cytoplasmic localization of NPM1 is not understood.\(^7\)
Here we elucidate for the first time another molecular mechanism leading to NPM1 cytoplasmic localization in AML. We described that the gene fusion NPM1-HAUS1 generates a putative chimeric protein with features that are very similar to NPM1m, such as identical size, disruption of tryptophan 288 and 290 and the generation of a novel NES. Moreover, we demonstrated that the chimeric protein NPM1-HAUS1 behaves exactly like mutated NPM1, in that both proteins localize to the cytoplasm and this localization is inhibited by the exportin-1 inhibitor leptomycin-B, suggesting that the novel NES generated by the fusion NPM1-HAUS1 is functional and responsible for its cytoplasmic localization.

The gene HAUS1 encodes a subunit of the human augmin complex that is involved in microtubule generation and mitotic spindle formation. This gene has not been studied in the context of cancer.

Corroborating the hypothesis that AML with gene fusion NPM1-HAUS1 is biologically similar to AML with mutated NPM1 is the fact that both patients studied here had CD34 negative blasts and tested positive for FLT3 ITD, with one patient also carrying DNMT3A S714C mutation, all features highly associated with NPM1 mutations in AML. Another report of AML with t(5;18) (q35;q21) also occurred in a patient with CD34 negative blasts, that presented gingival and lymph node involvement, both also associated with NPM1 mutated AML. Additionally, a further AML patient harboring a novel cytogenetic alteration ins(18;5)(q21.1;q31.2q35.1) also presented evidence of a juxtaposition of the genes NPM1 and HAUS1. Suggesting that this fusion can occur by diverse genomic mechanisms (translocations and insertions). While the molecular characterization was not possible in that case, it is worth noticing
that the patient also presented with CD34 negative blasts, FLT3 ITD and absence of NPM1 exon 12 mutations.

Although AML associated with t(5;18) (q35;q21) and NPM1-HAUS1 fusion is a rare entity and our findings suggest it is biologically similar to NPM1m AML, its impact on prognosis remains to be determined.

In conclusion, we have identified a novel mechanism of NPM1 cytoplasmic localization in AML, the gene fusion NPM1-HAUS1.
References


**Table 1.** Clinical and laboratorial features of the two patients with AML harboring the NPM1-HAUS1 fusion.

<table>
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<tr>
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<th>Patient 1</th>
<th>Patient 2</th>
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<tr>
<td><strong>Gender</strong></td>
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<td>CD13 CD33 CD117 HLA-DR</td>
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<td>18 months</td>
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<td>Refractory AML</td>
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*CT = chemotherapy; CR = complete remission*
Figures legends

Figure 1. Identification of the gene fusion NPM1-HAUS1 in Acute Myeloid Leukemia. A: Whole genome sequencing reads spanning the breakpoint between NPM1 intron 11 (chromosome 5) and HAUS1 intron 8 (chromosome 18). B: cDNA Sanger sequencing of the chimeric gene demonstrating the expression of an in frame fusion between NPM1 exon 11 to HAUS1 exon 9. C: Exon diagram demonstrating the putative chimeric transcript containing NPM1 exons 1 – 11 fused to HAUS1 exon 9 that contains the sequence encoding a nuclear export signal (NES). D: C-terminus of the proteins NPM1 WT, NPM1m and NPM1-HAUS1 illustrating the similarity between NPM1m and NPM1-HAUS1 in its identical size, the disruption of tryptophan 288 and 290, and the generation of a nuclear export signal motif (highlighted in yellow). Amino acids in cyan (NPM1m) and green (NPM1-HAUS1) represent residues not present in NPM1 WT.

Figure 2. NPM1-HAUS1 localizes both in the nucleus and cytoplasm through a Crm1-dependent transport. A: 293T cell line was transduced with lentiviral vectors containing NPM1 wild type fused to mCherry (upper panels), NPM1 with the AML type A (NPM1m) mutation fused to GFP (middle panels), or NPM1-HAUS1 fused to GFP (lower panels). Cells were plated on coverslips, fixed with 4% paraformaldehyde, and staining with DAPI for nucleus visualization. The localization of each construct is traced by the detection of mCherry and GFP. (63 X magnification). While NPM1 WT localizes exclusively in the nucleus, both NPM1m and NPM1-HAUS1 localize both in the nucleus and in the cytoplasm B: 293T cell line transduced with NPM1-HAUS1 (upper panels) and NPM1m (lower panels) were cultured in the presence of 4 µg/mL Leptomycin B for 4 hours, fixed and staining with DAPI. (100 X objective). The analysis was performed using Zeis LSM 710 Observer.Z1 microscope. On the presence of leptomycin, both NPM1m and NPM1-HAUS1 localize exclusively in the nucleus.