

Mutations and promoter methylation status of *NPM1* in myeloproliferative disorders

We determined mutations and promoter methylation status of *NPM1* using pyrosequencing in 199 samples of myeloid neoplasia including myeloproliferative disorders (MPD). The mutations were present in 4% of chronic myelomonocytic leukemia, but not in other MPD or myelodysplastic syndromes. Promoter methylation was rare, and was found in only three samples of MPD.

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Mutations in nucleophosmin 1 gene (*NPM1*, localized on 5q32) were found to be frequent events in acute myeloid leukemia (AML).¹⁻³ *NPM1* likely plays a role as a tumor suppressor in myeloid hematopoiesis, and its haploinsufficiency has been suggested.^{4,5} *NPM1* mutations have not been observed in myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), or lymphoid malignancies.¹ Here we describe a simple and sensitive screening method for *NPM1* mutation using a pyrosequencing assay, which has a detection limit of approximately 5% of mutant alleles.⁶ We applied this to 14

leukemic cell line samples (OCI/AML3, ALL1, BJAB, CEM, HEL, HL60, JTAG, Jurkat, K562, KG1, KG1a, ML1, Raji, and TF-1) and 199 samples from patients including 39 AML (including 11 with diploid karyotype and four with 5q or chromosome 5 deletion), 15 Ph-positive and 15 Ph-negative CML, 50 MDS (including ten with 5q or chromosome 5 deletion), 50 chronic myelomonocytic leukemia (CMML), 14 polycythemia vera (PV), 7 essential thrombocythemia (ET) and 9 myelofibrosis (MF). Samples from patients were obtained from peripheral blood or bone marrow mononuclear cells, and all patients gave their consent to the donation of samples. Furthermore, promoter methylation, which can cause gene silencing,⁷ of the *NPM1* gene was also analyzed.

The pyrosequencing assay for mutation analyses was the same as the one we had previously utilized for analysis of the *JAK2* mutation.⁶ First, exon 12 of the *NPM1* gene was amplified by polymerase chain reaction (PCR) using primers NPM1-F: 5'-TTAACTCTCTGGTGGTAGAATG-3' and biotinylated-NPM1-R: 5'-ACATTTATCAAACACGGTAGG-3'. Then the biotinylated strand was captured on streptavidin sepharose beads and annealed with a sequencing primer NPM1-S: 5'-TTTTCCAGGC-TATTCAAGAT-3'. Pyrosequencing was performed using PSQ HS 96 Gold SNP Reagents and the PSQ HS 96 pyrosequencing machine (Biotage, Uppsala, Sweden). Programmed polymorphic sites were set at nucleotides 959 (A/C), 960 (G/T) and 964 (G/C) to detect all previously reported mutation variants (Figure 1 A). Mutations are

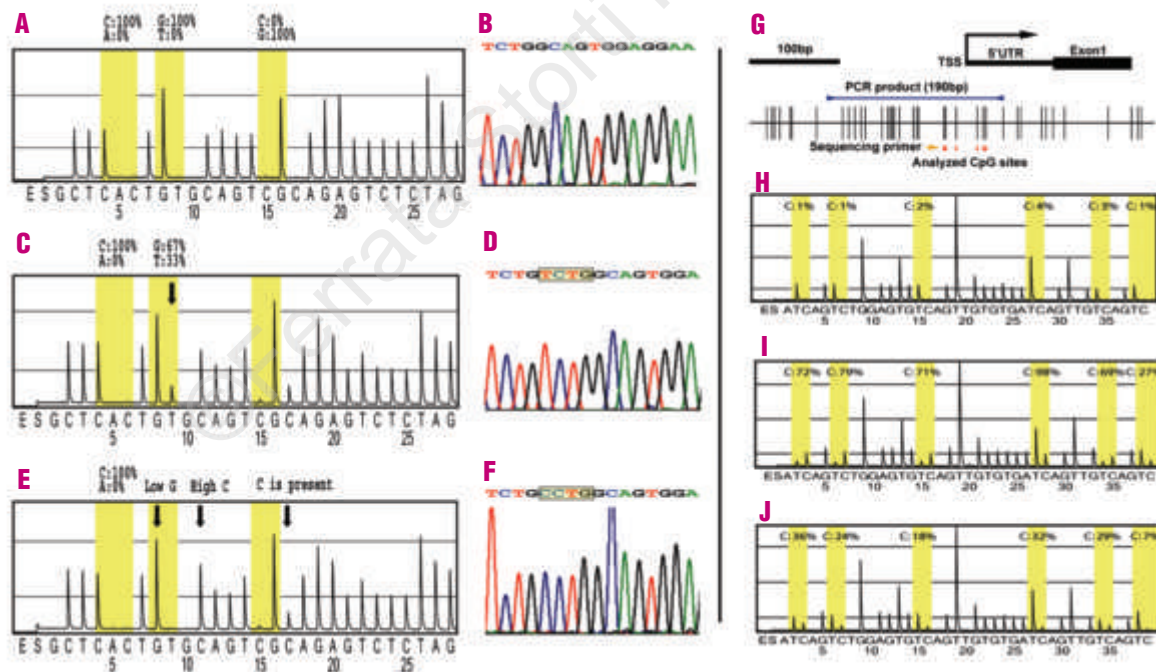


Figure 1. A-F. Mutation assays. Expected wild type sequence in this region is CTCTGGCAGTGGAGGAAGTCTCTTTAAG. **A.** A pyrogram of a normal sample showing only wild type allele. **B.** Cloning and sequencing confirmed a wild type sequence only. **C.** A pyrogram in a patient with CMML, showing an abnormal T peak at position 960, suggesting the presence of a mutant allele. **D.** Cloning and sequencing confirmed the presence of mutation A (960-961insTCTG). **E.** A pyrogram with multiple abnormal peaks in another patient with CMML, suggesting the presence of a mutant allele. **F.** Cloning and sequencing confirmed the presence of mutation D (960-961insCCTG). **G.** Schematic map of the promoter region. CpG sites at -21, -19, -8, 15, 22, and 24 bases from the transcription start site were analyzed. Vertical bars indicate CpG sites. TSS indicates the transcription start site; UTR: untranslated region. **H-J.** Methylation assays. The expected sequence in this region is YGYGGGAGTTTGYGTTTTTTTTTGGTGTGATTTGYGTYGTYG (Y=T or C). **H.** A pyrogram of a normal sample showing no methylation. **I.** A pyrogram of a SssI methylase treated normal control DNA (methylation positive control), showing an average methylation of 69% (average of six CpG sites). **J.** A pyrogram of a sample from a patient with myelofibrosis, showing an average methylation of 24%.

Table 1. Summary of the mutation and methylation analyses.

Disease	N	Prevalence of NPM1 mutation	Prevalence of NPM1 methylation
Leukemic cell lines	14	1 (OCI/AML3)	2 (CEM, ML1)
Acute myeloid leukemia	39	5 (13% of all cases, 45% of diploid cases)	0 (0%)
Chronic myeloid leukemia	15	0 (0%)	0 (0%)
Myelodysplastic syndrome	50	0 (0%)	0 (0%)
CMML	50	2 (4%)	0 (0%)
Ph-negative CML	15	0 (0%)	0 (0%)
Polycythemia vera	14	0 (0%)	1 (7%)
Essential thrombocytopenia	7	0 (0%)	0 (0%)
Myelofibrosis	9	0 (0%)	2 (22%)

CML: chronic myeloid leukemia; CMML: chronic myelomonocytic leukemia.

detected as abnormal pyrogram patterns (pyrosequencing peaks) compared to the wild type pattern. When a mutation was indicated, PCR was repeated using primers without the biotin tag, then cloned in a plasmid and sequenced at the M.D. Anderson Cancer Center DNA Core Facility using ABI Big Dye terminator cycle sequencing chemistry to confirm the mutation.

First, leukemic cell lines and primary AML samples were analyzed to confirm the validity of the mutation analysis. Among cell line samples, only OCI/AML3 showed the *NPM1* mutation, consistent with a previous report.¹² Among 39 AML samples, five with diploid karyotype showed *NPM1* mutations, all as forms of insertion between positions 959 and 960.⁸ The frequency of this mutation in AML patients with diploid karyotype is consistent with that in previous reports.¹⁻³ We did not observe mutations in other positions. The *NPM1* mutation was not observed in Philadelphia chromosome (Ph) positive-CML and MDS (excluding CMML) samples. Among patients with MPD, *NPM1* mutations were observed in two of 50 cases of CMML (Figure 1C, 1E) but in no cases of Ph-negative CML, ET, PV, or MF (Table 1). The patient with mutation A (960-961insTCTG) was a 78-year old male with CMML, with a white blood count of $15 \times 10^9/L$ and 13% peripheral monocytes. Bone marrow showed CMML, with 14% blasts and normal karyotype. This patient was treated with decitabine and achieved a complete remission, when *NPM1* mutation was undetectable. Mutation D (960-961insCCTG) was detected in a 77-year old female with CMML. Bone marrow showed 6% blasts and a normal karyotype. The woman had a peripheral white blood cell count of $6.4 \times 10^9/L$, with 1% blasts and 17% monocytes. Twelve months later, the patient developed AML with *NPM1* mutation D.

We used the bisulfite pyrosequencing method for methylation analyses.⁹ The promoter region of *NPM1* in bisulfite-treated DNA¹⁰ was amplified by PCR using primers NPM1-Bis-F: 5'-AAGGAGTGGGGTTGAAAAG-3' and biotinylated-NPM1-Bis-R: 5'-CCCTACTC-CAAAAACAACC-3'. After PCR, T/C polymorphisms, corresponding to unmethylated and methylated cytosines in the original DNA, at -21, -19, -8, 15, 22, and 24 bases from the transcription start site, were analyzed with

pyrosequencing,¹³ using sequencing primer NPM1-Bis-S: 5'-GAGATTTTTAGGGTTTATATATAAG-3' (Figure 1 G). The methylation percentage was calculated by the average of the degree of methylation at six CpG sites formulated in pyrosequencing. In cell lines, CEM and ML1 showed a low degree of methylation (average 29% and 36%, respectively). However, the *NPM1* expression assay (real-time PCR using Hs01576587_g1 [Applied Biosystems] and GAPDH as internal controls) showed no evidence of gene silencing, when compared to other cell lines (*data not shown*). In samples from patients, promoter hypermethylation was only observed in two cases with MF and one with PV (50%, 24% [Figure 1J] and 24%, respectively), who all had a diploid karyotype.

In conclusion, we screened samples of MDS and MPD for mutations of *NPM1*, and detected mutations in 4% of patients with CMML. *NPM1* mutations were not observed in MDS or other MPD. Promoter hypermethylation of *NPM1* is rare in myeloid neoplasms. *NPM1* mutations, methylation and 5q deletion were not found simultaneously, although our study included a limited number of patients. The significance of promoter hypermethylation needs to be investigated further.

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