A polymorphism in the 3'-untranslated region of the *NPM1* gene causes illegitimate regulation by microRNA-337-5p and correlates with adverse outcome in acute myeloid leukemia

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ABSTRACT

Nucleophosmin, encoded by *NPM1*, is a haploinsufficient suppressor in hematologic malignancies. *NPM1* mutations are mostly found in acute myeloid leukemia patients with normal karyotype and associated with favorable prognosis. A polymorphic nucleotide T deletion with unknown significance is present in the *NPM1* 3'-untranslated region. Here, we showed that the homozygous nucleotide T deletion was associated with adverse outcomes and could independently predict shortened survival in patients with *de novo* acute myeloid leukemia. Mechanistically, we demonstrated that the nucleotide T deletion created an illegitimate binding *NPM1* for *miR-337-5p*, which was widely expressed in different acute myeloid leukemia subtypes and inhibited *NPM1* expression. Accordingly, *NPM1* levels were found to be significantly reduced and correlated with *miR-337-5p* levels in patients carrying a homozygous nucleotide T-deletion genotype. Together, our findings uncover a microRNA-mediated control of *NPM1* expression that contributes to disease heterogeneity and suggest additional prognostic values of *NPM1* in acute myeloid leukemia.

Introduction

The *NPM1* gene is frequently altered in hematologic malignancies.[1] In acute myeloid leukemia (AML), *NPM1* is mainly disrupted by C-terminus mutations, causing aberrant cytoplasmic expression of the NPM1 protein.[2] *NPM1*-mutated AML has distinctive clinicopathological and molecular features. Most notably, the mutation is closely associated with normal karyotype and relatively favorable prognosis in the absence of *FLT3*-internal tandem duplication (ITD).[3] While genotyping the *NPM1* mutations, a polymorphic nucleotide T deletion (delT) at position 1,146 of the *NPM1* 3'-untranslated region (UTR) was reported in 60%-70% of AML patients.[4,5] However, the significance of this polymorphism was unclear. Previous studies have shown that polymorphisms in other genes, including *WT1* and *IDH1*, can affect AML prognosis in addition to mutations in the cognate genes.[6,7] Here, we revealed that the homozygous state of the *NPM1* delT polymorphism had important clinical and biological implications in AML involving an illegitimate microRNA (miRNA) regulation.

Design and Methods

Patients

We analyzed 149 adult and 70 childhood patients with *de novo* AML; therapy-related AML or AML arising from a prior myelodysplastic syndrome were excluded. Patients' characteristics are shown in *Online Supplementary Table S1*. All patients gave informed consent for the study, which was approved by the Joint CUHK-NTEC Clinical Research Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Patients with acute promyelocytic leukemia (APL) were excluded for survival analysis. Overall, complete survival data were available from 93 adult and 61 childhood patients with non-APL who had received treatment. All the 93 adult patients were treated with the standard cytarabine plus daunorubicin '7+3' induction chemotherapy regimen.[8] Patients who achieved complete remission (CR) were then given consolidation treatment stratified by cytogenetics. Patients with non-favorable cytogenetics were referred for assessment for allogeneic stem cell transplantation. Patients who were not eligible for transplantation and those with favorable cytogenetics were given high-dose cytarabine-based chemotherapy for consolidation.[9] Of the 61 pediatric patients, 41 were treated with a modified UK MRC AML 12 protocol[10] and 20 with the NOPHO-AML 2004 protocol,[11] as previously described. Two adult and 11 pediatric patients received transplantation as consolidation therapy. For these patients, survival data had been censored at the time of transplantation.

Normal bone marrow (BM) and peripheral blood samples were obtained from healthy donors who had no prior history of malignancy.

Cell culture

Cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum.

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Cytogenetic and mutational studies

Cytogenetics were classified into favorable, intermediate, and adverse according to the UK MRC classification.[12,13] Intermediate/adverse cytogenetics are hereafter collectively referred to as non-favorable. Patients with unknown cytogenetics were screened for the presence (favorable) or absence (non-favorable) of *RUNX1-ETO, CBFB-MYH11* and *PML-RARA* fusion transcripts.[10]

Mutation analysis of *FLT3*-ITD, *KIT* (exons 8 and 17), *NPM1* (C-terminus and the delT polymorphism), and *CEBPA* was performed as previously described.[10] Exon 4 of *IDH1* (R132) and *IDH2* (R140 and R172) were analyzed by direct sequencing. Primer sequences are provided in *Online Supplementary Table S2*.

Constructs, transfection, and luciferase reporter assays

Full-length *NPM1* 3'-UTR carrying the wild-type (pmirGLO-3'UTR-WT) or delT (pmirGLO-3'UTR-delT) genotype was cloned into the dual-luciferase pmirGLO vector (Promega), which co-expresses *Renilla* luciferase for normalization of transfection efficiency. pmirGLO-WT×3 and pmirGLO-delT×3 were prepared by cloning of the annealed oligonucleotides NPM1-WT×3-F/NPM1-WT×3-R and NPM1-delT×3-F/NPM1-delT×3-R (nucleotide sequences are provided in *Online Supplementary Table S2*) into pmirGLO. Pre-miR precursors were obtained from Life Technologies. Transfection was performed using Lipofectamine 2000 (Life Technologies). Luciferases were measured by Dual-Glo Luciferase Assay System (Promega) 24 h after transfection.

Quantitative RT-PCR (qRT-PCR)

RNA was extracted using TRIzol (Life Technologies). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using TaqMan assays (Life Technologies). *NPM1* levels were normalized to *GAPDH*, and miRNAs normalized to *RNU48*. Relative expression was calculated by $2^{-\Delta\Delta Ct}$.

Western blotting

Western blotting was performed as previously described using NPM1 and β-tubulin antibodies (Cell Signaling).[10]

Statistical analysis

Overall survival (OS) and relapse-free survival (RFS) were defined as previously described.[14] Kaplan-Meier curves were compared by log rank test. Multivariate Cox's regression analysis was performed to test the significance of the delT polymorphism with adjustment for other potential prognostic factors. Pearson's correlation was used to analyze correlations between continuous parameters. Two-sided $P<0.05$ was considered statistically significant. SPSS 13.0 was used for statistical analyses (SPSS).

Results and Discussion

Of the 149 adult AML patients, 105 carried the delT polymorphism (32 homozygous and 73 heterozygous). The polymorphism was also determined in 211 healthy individuals (54% males; median age 50 years, range 21-74 years) and the genotype frequency was found to be similar between the normal (38 homozygous and 102 heterozygous) and adult patient (P=0.603) cohort, suggesting that the polymorphism might not affect susceptibility to leukemia development. Among 93 adults with non-APL with survival data, 44 (47%) died during a mean follow up of 21 months. Seventy-one patients (76%) achieved CR of whom 27 (38%) relapsed. Patients carrying a homozygous delT genotype had higher relapse rates (59% *vs.* 31%; P=0.051) and significantly shortened OS (median 9 months *vs.* 12 months, P=0.016) and RFS (median 5 months *vs.* 12 months; P=0.007) than patients carrying a non-homozygous genotype (Figure 1 A and B). CR rates were similar between the two groups (74% *vs.* 77%, P=0.781). In multivariate analysis, the homozygous delT genotype (P=0.035), age (P=0.013), white blood cell count (P=0.006), cytogenetics (P=0.044), and *CEBPA* double mutation (P=0.004) were significant prognostic factors for OS (*Online Supplementary Table S3*). For RFS, the homozygous delT genotype (P=0.018), age (P=0.005), cytogenetics (P=0.026), and *CEBPA* double mutation (P=0.002) were significant factors.

Because elderly and young adult AML patients have different biological and clinical behaviors, we next restricted our analysis to younger adult patients who were 18-60 years old (n=80). Younger patients with homozygous delT had higher relapse rates (60% *vs.* 32%; P=0.07) and significantly reduced OS (median 10.5 months *vs.* 13 months; P=0.038) and RFS (median 7 months *vs.* 13 months; P=0.019) than patients with a non-homozygous genotype (Figure 1C and D). CR rates were similar between the two groups (83% *vs.* 81%, P=1.000). The homozygous delT genotype remained prognostic for poorer RFS (P=0.028) in multivariate analysis (*Online Supplementary Table S3*). Based on these findings, we further evaluated the impact of the homozygous delT genotype on younger adult patients with different cytogenetic and molecular features. Despite the limited sample size, a trend or significant association of homozygous delT with poorer RFS was observed in all the subgroups analyzed (*Online Supplementary Figure S1*). The prognostic impact of the delT polymorphism in elderly patients was not determined because of the small number of patients over the age of 60 years.

The delT polymorphism was detected in 44 (12 homozygous and 32 heterozygous) of 70 childhood AML patients. Among 61 non-APL patients with survival data, 12 (20%) died during a mean follow up of 54 months. Fifty-nine patients (97%) achieved CR of whom 16 (27%) relapsed. The homozygous delT genotype had no significant impacts on OS, RFS, and CR rates in the entire cohort. However, when patients with *FLT3*-ITD (n=7) and *KIT* mutations (n=5), the two molecular markers reported to correlate with high relapse risk in childhood AML,[15] were excluded, a significant association between homozygous delT and poorer RFS (P=0.032) was noted; the association with OS was not statistically significant (P=0.106).

The delT polymorphism showed no significant correlation with any clinicopathological or molecular parameters in the adult and childhood patient cohorts (*Online Supplementary Table S4*).

Polymorphisms within 3'-UTR might modify miRNA-mediated gene regulation. Using the PITA algorithm,[16] we found that the delT created three putative binding sites for *miR-337-5p*, *miR-887*, and *miR-553* (Figure 2A). To investigate the potential relevance of these miRNAs in regulating *NPM1*, we first examined their expression in BM from patients with different AML subtypes. All the 16 AML samples and 10 normal BM expressed detectable levels of *miR-337-5p* and *miR-887* (Figure 2B). In contrast, none of the BM examined (16 patients and 11 normal) showed *miR-553* expression (*data not shown*). Thus, *miR-337-5p* and *miR-887* were selected for functional validation using luciferase reporter assays in HeLa which expressed barely detectable levels of the two miRNAs (*data not shown*). Co-

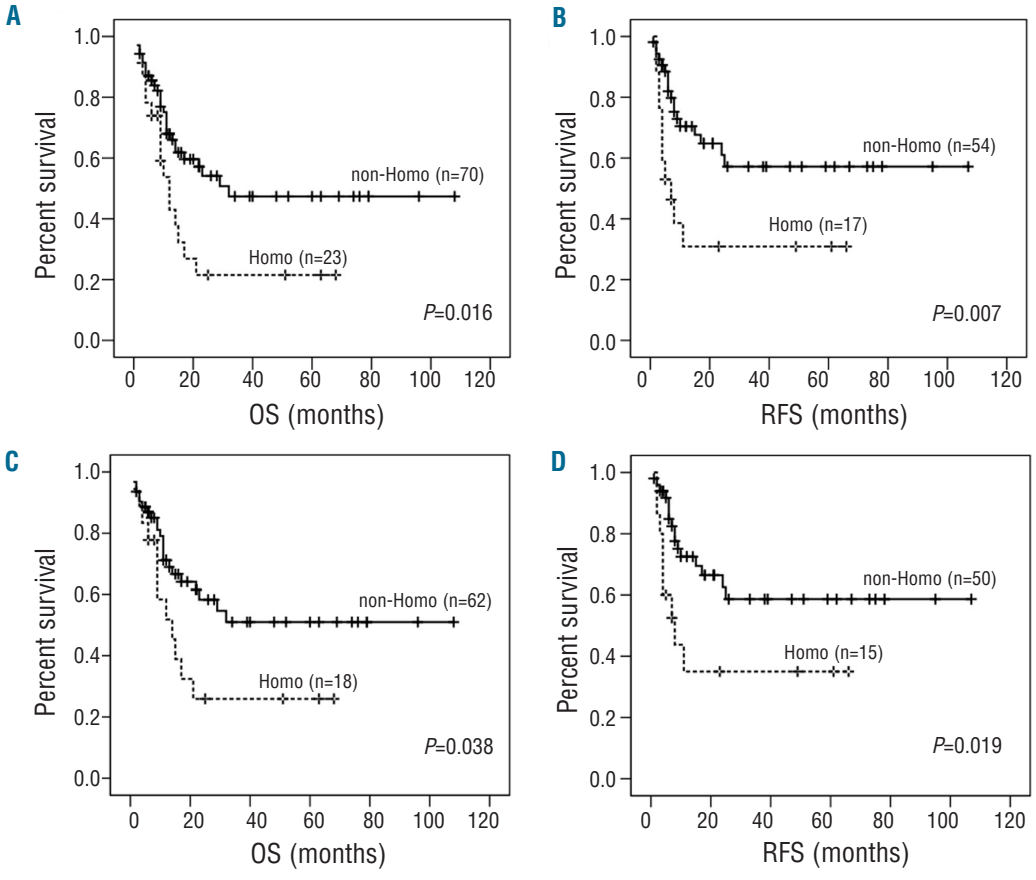

Figure 1. The homozygous *NPM1* 3'-UTR delT genotype was associated with inferior outcomes in AML patients. Kaplan-Meier analysis of OS and RFS based on the delT genotype in the entire adult AML cohort (A and B) or younger adult patients aged 18 to 60 years (C and D). Patients failed to achieve CR were omitted in the RFS analysis. Homo, homozygous; non-Homo, non-homozygous.

transfection of pre-miR-337-5p exerted a dose-dependent repressive effect on the *NPM1* 3'-UTR harboring the delT (Figure 2C and *Online Supplementary Figure S2*). In contrast, no effect on the 3'-UTR-delT construct was observed when pre-miR-887 was used. Moreover, co-transfection of these two miRNA precursors had no effect on the wild-type 3'-UTR construct (Figure 2C). To validate this finding, we created two additional constructs, each containing three tandem copies of the delT (pmirGLO-delT×3) or wild-type (pmirGLO-WT×3) sequences. Co-transfection with pre-miR-337-5p but not pre-miR-887 drastically repressed the pmirGLO-delTx3 construct (Figure 2C). We next transfected K562, U937 and HeLa cells with pre-miR-337-5p to investigate the effect on endogenous *NPM1* expression. These cell lines carried the homozygous delT, heterozygous delT and wild-type genotypes, respectively, and expressed barely detectable levels of *miR-337-5p* (*data not shown*). Transfection of pre-miR-337-5p reduced both *NPM1* mRNA and protein levels in K562 cells harboring the homozygous delT polymorphism (Figure 2D). In contrast, no apparent effect on *NPM1* expression was observed in U937 and HeLa cells. Collectively, these findings indicate that the delT caused illegitimate repression of *NPM1* by *miR-337-5p*. We next compared *NPM1* transcript levels in AML patients with or without homozygous delT. Since *NPM1* is ubiquitously and abundantly expressed,[1] only patients whose BM contained at least 80% of blasts were selected for analysis. As shown in Figure 2E, *NPM1* levels were significantly reduced in the homozygous delT group as compared to the non-homozygous group. Importantly, those patients with lower *NPM1* expression were also found to have poorer outcomes than those with higher *NPM1* expression (*Online Supplementary Figure S3*). Notably, a significant inverse relationship between *NPM1* and *miR-337-5p* levels was observed in patients carrying the homozygous delT genotype, but not in patients carrying the other genotypes (Figure 2F).

NPM1-mutated AML has been found to have a distinctive miRNA expression profile,[17-19] however, whether *NPM1* is subjected to miRNA regulation is unclear. In this study, we demonstrated that a polymorphic delT in the *NPM1* 3'-UTR generated an illegitimate binding site for *miR-337-5p*. Analysis of the entire *NPM1* mRNA sequence revealed no further *miR-337-5p* site, highlighting the significance of the polymorphism in *miR-337-5p*-mediated *NPM1* regulation. Moreover, the use of other databases failed to reveal additional miRNAs targeting this delT site. 6-mer seed-matched sites typically have lower efficacy[20] and this might explain the modest *NPM1* repression by *miR-337-5p*. It is worth noting that the *miR-337-5p*-mediated *NPM1* regulation might be cell context-dependent as we failed to observe similar correlations of *NPM1* and *miR-337-5p* expression in normal peripheral blood samples (*Online Supplementary Figure S4*). Indeed, we noticed that the normal samples had markedly higher *NPM1* levels (approx. 7.3-fold) than the AML patient samples while *miR-337-5p* expression was similar between the two groups. Since the endogenous level of the target mRNA is an important determinant of miRNA regulation,[21] these findings implicate potential differential regulation of *NPM1* expression in normal and AML leukemic cells. To

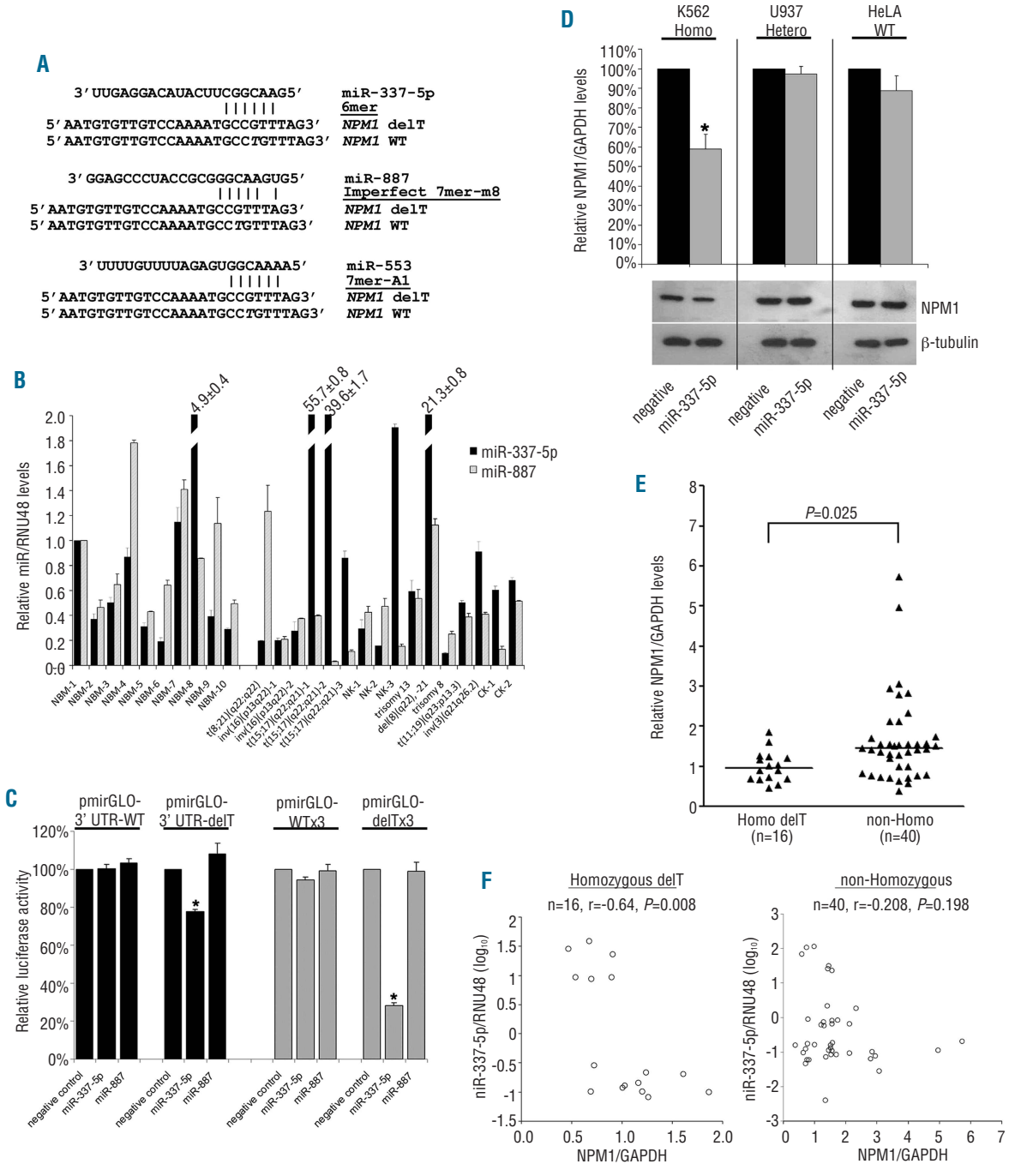

Figure 2. The homozygous delT polymorphism in *NPM1* 3'-UTR caused illegitimate regulation by *miR-337-5p*. (A) Sequence alignment of *miR-337-5p*, *miR-887*, and *miR-553* with the *NPM1* 3'-UTR delT sequence. The delT in the wild-type (WT) sequence is in *italics*. The type of microRNA seed match[20] is indicated. (B) Expression of *miR-337-5p* and *miR-887* in BM samples from 16 adult AML patients and 10 normal individuals. Relative expression was compared to NBM-1. The cytogenetics of each patient is indicated. NK: normal karyotype; CK: complex karyotype; NBM: normal BM. Results are expressed as mean±SE from triplicate assays. The column bar of four outliners was truncated and the relative levels are shown. (C) *NPM1* 3'-UTR luciferase constructs (0.2 μg) were co-transfected with pre-miR-337-5p or pre-miR-887 precursor at a final concentration of 100nM into HeLa cells. Transfection with the same amount of pre-miR negative control was done in parallel. Results are presented as relative luciferase activity by comparing the normalized firefly luciferase activity of the construct co-transfected with pre-miR precursor to that co-transfected with the negative control. Results are expressed as mean±SE from at least triplicate assays. *indicates $P<0.05$. (D) K562, U937, and HeLa cells were transfected with 100nM of pre-miR-337-5p or pre-miR negative control and *NPM1* expression was determined 72 h after the transfection. Flow cytometric analysis of FAM-labeled pre-miR negative control revealed a transfection efficiency of 91-97% for the three cell lines (*data not shown*). *NPM1* mRNA levels (upper panel) were compared between the pre-miR-337-5p and control transfection. qRT-PCR results are presented as mean±SE from triplicate assays. *$P<0.05$. Lower panel, NPM1 levels were examined by Western blot and β-tubulin was used as loading control. Representative blots from repeated experiments are shown. The delT genotype in each cell line is indicated. Homo: homozygous delT; Hetero, heterozygous delT. (E) Comparison of *NPM1* mRNA levels in adult AML patients with or without the homozygous delT polymorphism. Each triangle represents one patient and the number of patients in each group is shown. Horizontal lines indicate the mean *NPM1/GAPDH* ratio. (F) Correlation of *NPM1* with *miR-337-5p* levels in adult AML patients with different *NPM1* 3'-UTR genotypes. Each circle represents one patient and the number of patients and the Pearson's correlation coefficient (r) in each group is shown. For (E) and (F), all patients had at least 80% blast counts in their BM. The cohort includes 20 patients with *PML-RARA* and 36 patients with a non-favorable karyotype (31 intermediate, 3 adverse and 2 unknown). Relative expression levels in each patient were determined in triplicates and compared to U937 (*NPM1*) and NBM-1 (*miR-337-5p*).

date, very little is known about the role of *miR-337-5p*. It was shown that the miRNA might be involved in the regulation of the tyrosine kinase gene LYN in B-cell chronic lymphocytic leukemia.[22]

The similar frequencies of the delT polymorphism between adult AML patients and normal adults suggested that the polymorphism might not predispose to leukemia. Likewise, the cytoplasmic *NPM1* mutant also failed to cause AML in transgenic mice, suggesting the need for co-operating mutations.[23] On the other hand, our findings indicated that the delT in homozygous state had negative impacts on AML outcome. Although the mechanisms by which the delT impacts outcome are unclear, it is possible that the delT-associated reduction in *NPM1* expression may cause centrosome amplification and genomic instability and promote tumorigenicity of the leukemic cells.[1] Another potential mechanism is that the delT-harboring *NPM1* mRNA may act as competing endogenous RNA (ceRNA) to compete with other mRNA targets for *miR-337-5p* binding, thereby regulating their function in trans and perturbing normal miRNA regulation.[24] Larger prospective studies are needed to further evaluate the prognostic value of the delT polymorphism and *NPM1* expression as well as their interaction with other mutations in AML patients.

In summary, we demonstrated the impact of genetic variations in an miRNA network involving fine control of a tumor suppressor and identified a potential poor-risk marker in AML.

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Authorship and Disclosures

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