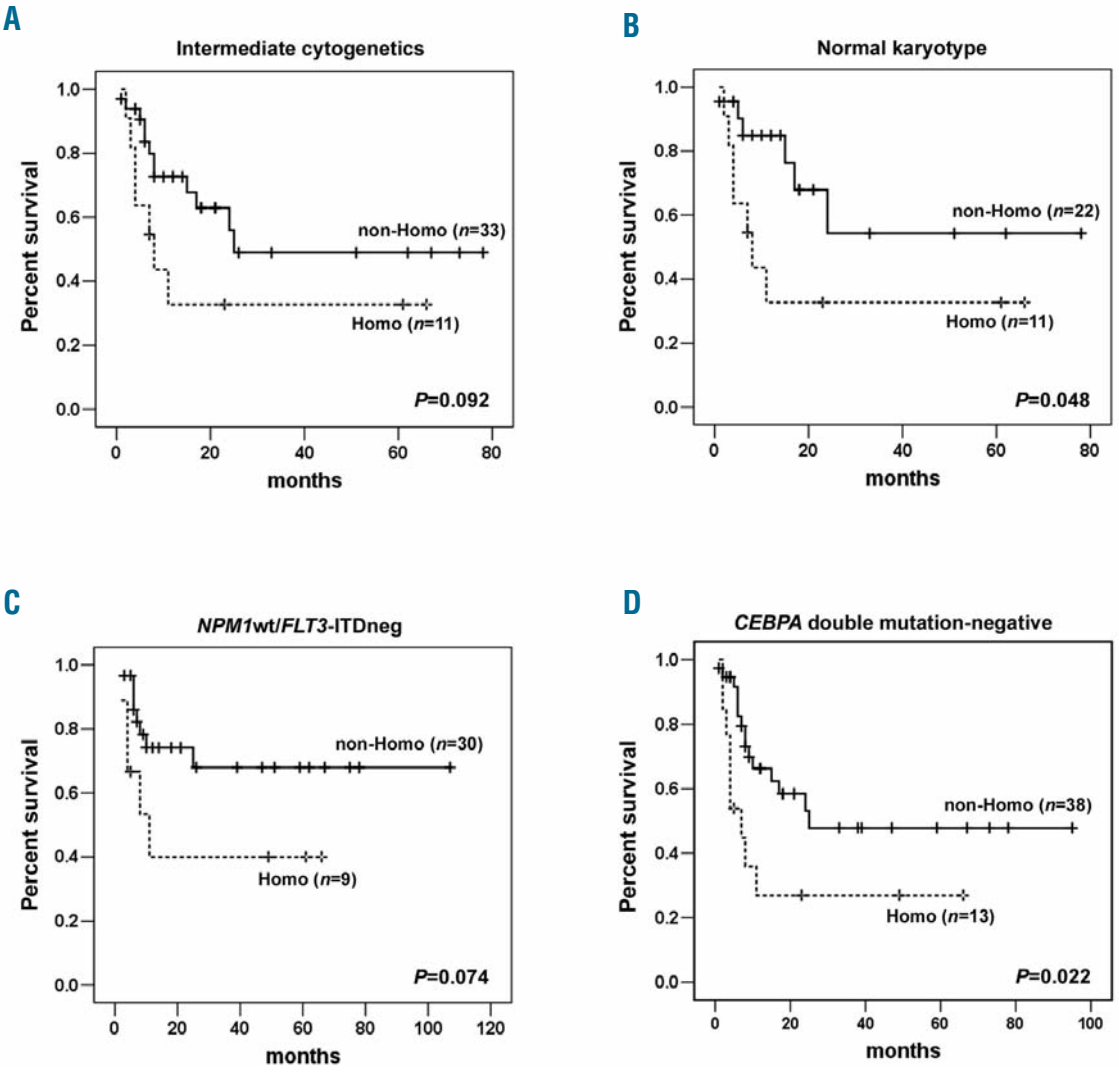
A polymorphism in the 3'-untranslated region of the *NPM1* gene causes illegitimate regulation by microRNA-337-5p and correlates with adverse outcome in acute myeloid leukemia

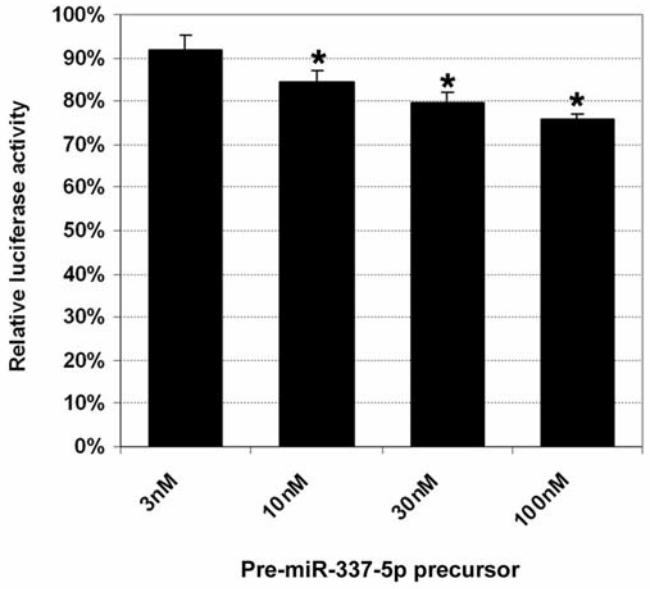
Chi Keung Cheng,[1] Tsz Ki Kwan,[1] Chi Ying Cheung,[1] Kitty Ng,[1] Pei Liang,[1] Suk Hang Cheng,[1] Natalie P. H. Chan,[1] Rosalina K. L. Ip,[1] Raymond S. M. Wong,[2] Vincent Lee,[3] Chi Kong Li,[3] Sze Fai Yip,[4] and Margaret H. L. Ng[1,5]

[1]Department of Anatomical and Cellular Pathology, [2]Department of Medicine and Therapeutics, [3]Department of Pediatrics, Prince of Wales Hospital, The Chinese University of Hong Kong, [4]Department of Medicine, Tuen Mun Hospital, Hong Kong, and [5]State Key Laboratory in Oncology in South China, The Chinese University of Hong Kong

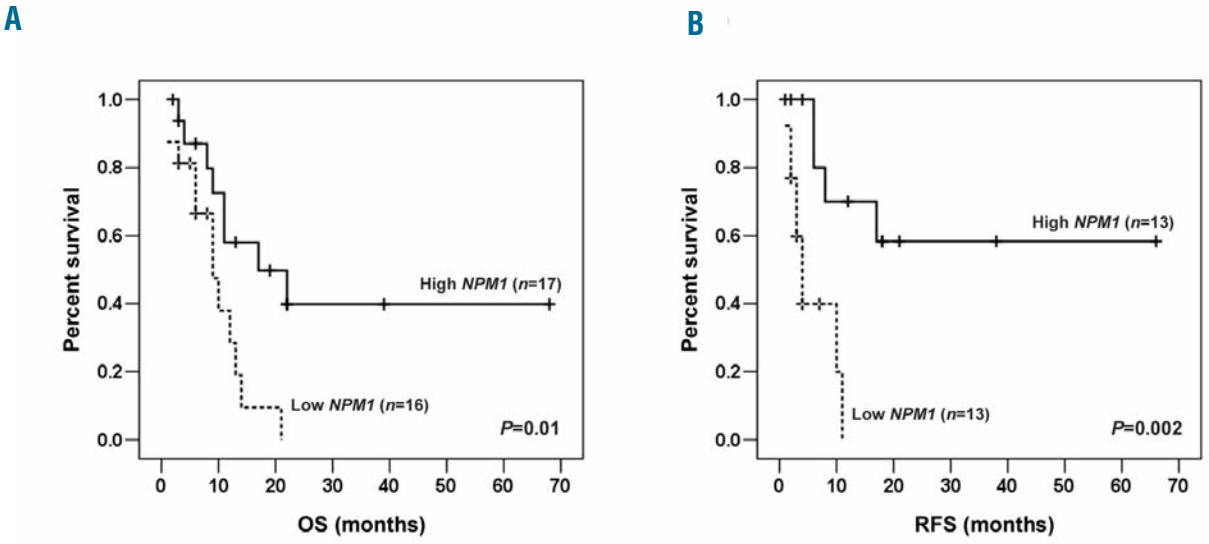
 doi:10.3324/haematol.2012.073015



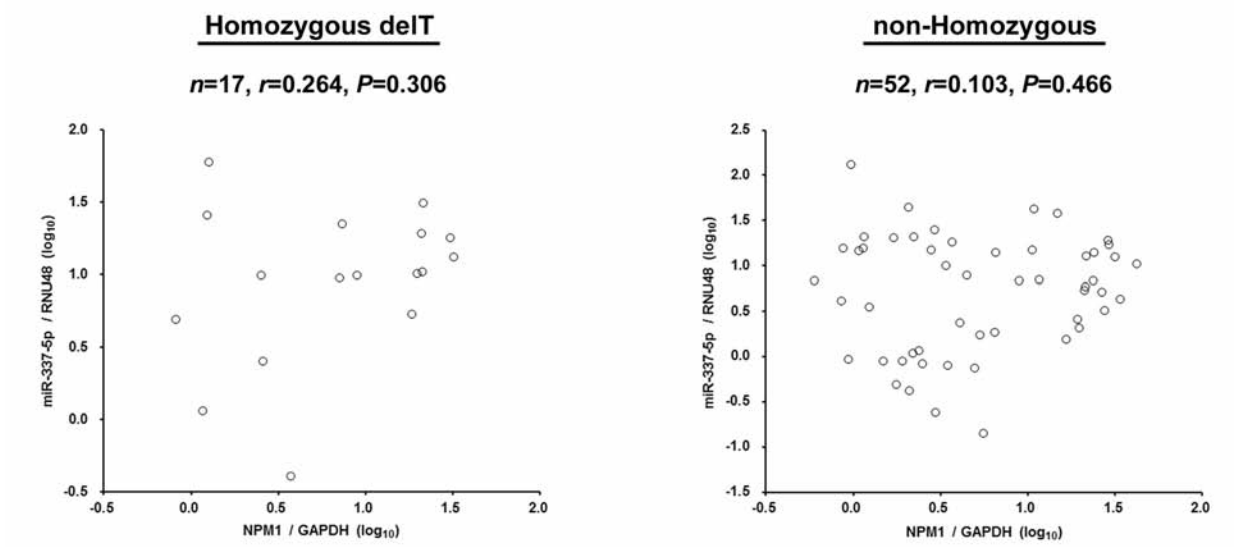
Online Supplementary Figure S1. Kaplan-Meier analysis of RFS based on the *NPM1* 3'-UTR delT genotype in younger adult non-APL patients (aged 18-60 years) with intermediate cytogenetics (normal and abnormal karyotypes) (A), normal karyotype (B), the *NPM1wt/FLT3*-ITDneg genotype (C), the absence of *CEBPA* double mutation (D). Homo, homozygous; non-Homo: non-homozygous. The number of patients with favorable or adverse cytogenetics was small and thus not separately analyzed.



Online Supplementary Figure S2. Dose-dependent inhibition of the *NPM1* 3'-UTR construct containing the delT by *miR-337-5p*. The pmirGLO-3'UTR-delT construct (0.2 μg) was co-transfected with different concentrations of the pre-miR-337-5p precursor 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-337-5p precursor to that co-transfected with the negative control. Results are expressed as mean±SE from at least triplicate assays. **P*<0.05.



Online Supplementary Figure S3. Kaplan-Meier analysis of OS (A) and RFS (B) based on *NPM1* mRNA levels in adult non-APL patients who had ≥80% blasts in their bone marrow (refer to Figure 2E and 2F) with survival data. *NPM1* levels were dichotomized at the median and divided into high and low expression groups.



Online Supplementary Figure S4. Correlation of *NPM1* with miR-337-5p levels in 69 normal peripheral blood samples from healthy donors (52% males; median age 49 years, range=17-85) with different *NPM1* 3'-UTR genotypes. Each circle represents one sample and the number of samples and the Pearson's correlation coefficient (*r*) in each group is shown. Relative expression levels in each sample were determined in triplicates and compared to U937 (*NPM1*) and NBM-1 (*miR-337-5p*).

Online Supplementary Table S1. Characteristics of the adult and childhood AML patient cohorts.

Parameters	Adult AML (n=149)	Childhood AML (n=70)
Mean age, years (range)	48.5 (18-85)	8.2 (0.3-17)
Sex, n (% males)	79 (53%)	39 (56%)
Mean hemoglobin, g/dL (range)	8.3 (3.5-14.4)	7.5 (4.3-13.2)
Mean platelets, 10^9/L (range)	60.3 (2-338)	61 (6-195)
Mean WBC, 10^9/L (range)	37.6 (0.6-282.2)	37.1 (0.9-295.7)
FAB subtypes, n		
M0	1	1
M1	30	9
M2	36	18
M3	27	7
M4	24	7
M5	26	15
M6	4	1
M7	0	10
Unknown	1	2
Cytogenetics, n (%)		
Favorable	43 (29%)	25 (36%)
Intermediate-normal karyotype	61 (41%)	20 (29%)
Intermediate-abnormal karyotype	24 (16%)	17 (24%)
Adverse	9 (6%)	8 (11%)
Unknown	12 (8%)	0 (0%)
Molecular markers, n (%)		
FLT3-ITD	26 (17%)	9 (13%)
KIT mutation	3 (2%)	5 (7%)
CEBPA double mutation	18 (12%)	4 (6%)
NPM1 mutation	31 (21%)	3 (4%)
NPM1 3'-UTR homozygous delT	32 (21%)	12 (17%)
IDH1 mutation	5 (3%)	1 (1%)
IDH2 mutation	12 (8%)	2 (3%)

WBC: white blood cell; FAB: French-American-British.

Online Supplementary Table S2. Sequences of oligonucleotides used.

Name	Sequence (5' to 3')*	Purpose	Size of PCR product (bp)
IDH1-Ex4-F	AGAGCCTTCGCTTTCTGCAT	IDH1 genotyping	
IDH1-Ex4-R	GCAAAATCACATTATTGCCAAC	IDH1 genotyping	430
IDH2-Ex4-F	GCTGCAGTGGGACCACTATT	IDH2 genotyping	
IDH2-Ex4-R	CTAGGCGAGGAGCTCCAGT	IDH2 genotyping	261
NPM1-WT×3-F	AAACTAGCGGCCGCTGTC*CAAAATGCCTGTTTAGTT TTTAATGTCCAAAATGCCTGTTTAGTTTTTAATGTCCAA AATGCCTGTTTAGTTTTTAAT*	Luciferase reporter construct	
NPM1-WT×3-R	CTAGA*TTAAAAACTAAACAGGCATTTTGGACATTAAAA ACTAAACAGGCATTTTGGACATTAAAAACTAAACAGG CATTTTGGACA*GCGGCCGCTAGTTT	Luciferase reporter construct	
NPM1-delT×3-F	AAACTAGCGGCCGC*TGTCCAAAATGCCGTTTAGTTT TTAATGTCCAAAATGCCGTTTAGTTTTTAATGTCCAAA ATGCCGTTTAGTTTTTAA*T	Luciferase reporter construct	
NPM1-delT×3-R	CTAGA*TTAAAAACTAAACGGCATTTTGGACATTAAAAA CTAAACGGCATTTTGGACATTAAAAACTAAACGGCATT TTGGACA*GCGGCCGCTAGTTT	Luciferase reporter construct	

**Italic sequences represent the three tandem copies of the NPM1 3'-UTR wild-type and delT sequences. Underlined sequences indicate the miR-337-5p seed match created by the delT.*

Online Supplementary Table S3. Results of multivariate analysis for overall and relapse-free survival.

Entire cohort (n=93)		OS			RFS	
Variable†	*P*	HR	95% CI	*P*	HR	95% CI
Age	0.013*	2.799	1.241-6.31	0.005*	5.809	1.698-19.871
WBC count	0.006*	2.461	1.291-4.692	0.143	1.782	0.822-3.863
Cytogenetics	0.044*	3.051	1.033-9.012	0.026*	4.163	1.187-14.6
CEBPA	0.004*	0.119	0.028-0.509	0.002*	0.088	0.019-0.404
NPM1/FLT3-ITD	0.114	0.455	0.171-1.209	0.144	0.46	0.163-1.304
NPM1 3'-UTR delT	0.035*	1.979	1.050-3.731	0.018*	2.653	1.186-5.936
IDH1	0.23	0.287	0.037-2.2	0.161	0.233	0.03-1.79
IDH2	0.608	0.797	0.335-1.895	0.087	0.246	0.049-1.223

Younger adult patients (18-60 years) (n=80)		OS			RFS	
Variable	*P*	HR	95% CI	*P*	HR	95% CI
WBC count	0.064	1.965	0.962-4.015	0.207	1.709	0.743-3.931
Cytogenetics	0.014*	4.636	1.36-15.81	0.014*	6.48	1.453-28.892
CEBPA	0.005*	0.056	0.007-0.419	0.006*	0.057	0.007-0.442
NPM1/FLT3-ITD	0.026*	0.188	0.043-0.821	0.142	0.427	0.137-1.33
NPM1 3'-UTR delT	0.104	1.839	0.882-3.833	0.028*	2.639	1.113-6.259
IDH1	0.22	0.27	0.033-2.186	0.22	0.266	0.032-2.203
IDH2	0.754	0.854	0.317-2.296	0.386	0.521	0.119-2.278

WBC: white blood cell; HR: hazard ratio; CI: confidence interval. †Age: > 60 versus *≤ 60 years old; WBC: above* versus *below median; Cytogenetics: non-favorable* versus *favorable;* CEBPA*: double mutation* versus *others;* NPM1/FLT3-*ITD:* NPM1^{mut}/FLT3-*ITD*^{pos} versus *other subtypes;* NPM1 *3'-UTR delT: homozygous* versus *non-homozygous;* IDH1 *and* IDH2*: positive* versus *negative for mutation. * Statistically significant. Hazard ratio > 1 (or <1) indicates a higher risk (or lower risk) of an event for the first category listed for dichotomous variables.*

Online Supplementary Table S4. Correlation analysis of the *NPM1* delT polymorphism* with different clinicopathologic and molecular markers.

Parameters	Adult AML (n=149) Homozygous (n=32)	Adult AML (n=149) Non-homozygous (n=117)	P	Childhood AML (n=70) Homozygous (n=12)	Childhood AML (n=70) Non-homozygous (n=58)	P
Mean age, years	52.1	47.5	0.114	8.7	8.1	0.736
Sex, n (% males)	18 (56%)	61 (52%)	0.695	9 (75%)	30 (52%)	0.204
Mean hemoglobin, g/dL	8.6	8.2	0.325	8.5	7.4	0.094
Mean platelets, 10^9/L	75.1	56.2	0.084	60.2	60.9	0.959
Mean WBC, 10^9/L	45.3	35.5	0.362	54.3	33.5	0.218
FAB subtypes, n			0.972			0.914
M0	0	1		0	1	
M1	7	23		2	7	
M2	8	28		3	15	
M3	6	21		1	6	
M4	5	19		2	5	
M5	6	20		2	13	
M6	0	4		0	1	
M7	0	0		1	9	
Unknown	0	1		1	1	
Cytogenetics, n (%)			0.484			0.715
Favorable	9 (28%)	34 (29%)		4 (33%)	21 (36%)	
Intermediate-normal karyotype	16 (50%)	45 (38%)		5 (42%)	15 (26%)	
Intermediate-abnormal karyotype	5 (16%)	19 (16%)		2 (17%)	15 (26%)	
Adverse	0 (0%)	9 (8%)		1 (8%)	7 (12%)	
Unknown	2 (6%)	10 (9%)		0 (0%)	0 (0%)	
Molecular markers, n (%)						
FLT3-ITD	7 (22%)	19 (16%)	0.441	2 (17%)	7 (12%)	0.646
KIT mutation	0 (0%)	3 (3%)	1.000	1 (8%)	4 (7%)	1.000
CEBPA double mutation	3 (9%)	15 (13%)	0.765	0 (0%)	4 (7%)	1.000
NPM1 mutation	5 (16%)	26 (22%)	0.473	0 (0%)	3 (5%)	1.000
IDH1 mutation	2 (6%)	3 (3%)	0.292	0 (0%)	1 (2%)	1.000
IDH2 mutation	3 (9%)	9 (8%)	0.721	1 (8%)	1 (2%)	0.316

**For both the adult and childhood AML cohorts, no significant correlation (P<0.05) with the parameters was observed when comparisons were made between wild-type and non-wild-type genotypes.*