

Clinical and functional implications of microRNA mutations in a cohort of 935 patients with myelodysplastic syndromes and acute myeloid leukemia

Felicitas Thol,^{1*} Michaela Scherr,^{1*} Aylin Kirchner,¹ Rabia Shahswar,¹ Karin Battmer,¹ Sofia Kade,¹ Anuhar Chaturvedi,¹ Christian Koenecke,¹ Michael Stadler,¹ Uwe Platzbecker,² Christian Thiede,² Thomas Schroeder,³ Guido Kobbe,³ Gesine Bug,⁴ Oliver Ottmann,⁴ Wolf-Karsten Hoffmann,⁵ Nicolaus Kröger,⁶ Walter Fiedler,⁷ Richard Schlenk,⁸ Konstanze Döhner,⁸ Hartmut Döhner,⁸ Jürgen Krauter,^{1,9} Matthias Eder,¹ Arnold Ganser,¹ and Michael Heuser¹

**These authors contributed equally*

¹Department of Hematology, Hemostasis, Oncology and HSCT, Hannover Medical School, Hannover; ²Medizinische Klinik und Poliklinik I, Universitätsklinikum Carl Gustav Carus, Dresden; ³Klinik für Hämatologie, Onkologie und Klinische Immunologie, Heinrich Heine Universität, Düsseldorf; ⁴Department of Medicine, Hematology/Oncology, University Hospital, Frankfurt; ⁵Department of Hematology and Oncology, University Hospital Mannheim; ⁶Department of Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, Hamburg; ⁷Department of Medicine II, Oncological Center, Hubertus Wald University Cancer Center, University Hospital Hamburg-Eppendorf, Hamburg; ⁸Department of Internal Medicine III, University of Ulm; and ⁹Klinikum Braunschweig, Germany

Correspondence: thol.felicitas@mh-hannover.de
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Clinical and functional implications of microRNA mutations in a cohort of 935 patients with MDS and AML

Felicitas Thol^{1*}, Michaela Scherr^{1*}, Aylin Kirchner¹, Rabia Shahswar¹, Karin Battmer¹, Sofia Kade¹, Anuhar Chaturvedi¹, Christian Koenecke¹, Michael Stadler¹, Uwe Platzbecker², Christian Thiede², Thomas Schroeder³, Guido Kobbe³, Gesine Bug⁴, Oliver Ottmann⁴, Wolf-Karsten Hofmann⁵, Nicolaus Kröger⁶, Walter Fiedler⁷, Richard Schlenk⁸, Konstanze Döhner⁸, Hartmut Döhner⁸, Jürgen Krauter^{1,9}, Matthias Eder¹, Arnold Ganser¹, Michael Heuser¹

Supplemental data

Supplementary methods

Mutational analysis

The following genes were analysed for mutations in AML patients: *FLT3-ITD*, *NPM1*, *CEBPA*, *DNMT3A*, *TET2*, *IDH1*, *IDH2*, *MLL-PTD*, *WT1*, *NRAS*, *SETBP1*, *STAG1*, *STAG2*, *RAD21*, *SMC1A*, *SMC3*, and the fusion genes *CBFB/MYH11*, *RUNX1/RUNX1T1*, *MLL/MLLT3*, *NUP98/NSD1*.

The following genes were analysed for mutations in MDS patients: *ASXL1*, *DNMT3A*, *IDH1*, *IDH2*, *RUNX1*, *NRAS*, *TP53*, *NPM1*, *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *SETBP1*.

Gene expression analysis

Real-time reverse-transcriptase-polymerase chain reaction (RT-PCR) was performed with patient-derived RNA using RNU48 as an endogenous control (assay ID 4427975, Life Technologies, Darmstadt, Germany). MiR-142-3p and miR-142-5p expression levels were quantified using the TaqMan Gene Expression Assay (Life

Technologies, assay ID: 4440887 for hsa-miR-142-3p and 4427975 for hsa-miR-142-5p) in CN-AML patients. For expression analysis, we dichotomized the patient cohort into two groups according to expression below or above the median expression of miR-142-3p/RNU48 and miR-142-5p/RNU48 values.

Construction of lentiviral vectors

The retroviral MDH1-miR-142-PGK-GFP vector was obtained from Addgene. To generate the lentiviral vector pdc-H1-miR-142-3p-SEW, the plasmid pdc-SEW (pdc: plasmids resulting in double-copy proviruses) was digested with SnaBI and treated with bacterial alkaline phosphatase. The H1-miR-142-3p cassette was excised from MDH1-miR-142-PGK-GFP with EcoR//BamHI, the cohesive ends were filled in using Klenow and ligated with the vector-fragment. Site-directed mutagenesis of the miR-142-3p seed sequence at positions 54 to 57 was carried out using the Quick-change mutagenesis kit (Stratagene, Heidelberg, Germany) in which the pdc-H1-miR-142-3p-SEW was used as template. The isolated clones were verified by DNA sequencing. Lentiviral constructs encode GFP (green fluorescent protein) as a reporter gene.

The preparation of recombinant lentiviral supernatants and lentiviral transductions were performed as described earlier.(1)

Immunoblotting

Whole cell lysates were prepared with lysis buffer (20mM HEPES, pH 7.5, 0.4 M NaCl; 1 mM EDTA, 1 mM EGTA, 1mM DTT) supplemented with mini complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Bioscience, Uppsala, Sweden). Membranes

were incubated with the following antibodies according to the manufacturer's protocol: Anti-cyclin T2 (ab50979) from Abcam, GAPDH (cs2118), and MAP3K7IP2/TAB2 (cs3745) from Cell Signaling Technology; Chemiluminescence was used for visualization using the ECL Western blotting detection reagents (Amersham Biosciences) according to the manufacturer. Densitometric analysis of x-ray films was performed using VersaDoc 3000 Imaging system (Bio-Rad) and 1-D analysis software Quantity One Version 4.6.5 (Bio-Rad). The intensity ratio of the protein of interest band to the GAPDH band (loading control) was calculated to measure changes in protein levels.

Supplemental tables

Supplementary Table S1. Primers used for gene amplification.

Primer for microRNAs		Sequence 5' to 3'
miR-142	F	CTTGGAGCAGGAGTCAGGAG
	R	CTCACCTGTCACACGAGGTC
miR-632	F	CATTTCGCATGCCCTTAG
	R	CCAAATCCAGGCCTCCTAGT
miR-891	F	GGTTCCCAAAGAGTCTACAAATG
	R	TGCTGCTACCTGTCCTCTGA

Supplementary Table S2: Comparison of target genes of wildtype and mutated miR-142-3p through analysis with targetscan (release 5.2: June 2011) (www.targetscan.org/vert_50/).

	wildtype miR-142-3p	pos54U_C	pos55A_G	pos56G_U	pos57U_C
Conserved targets (no.)	250	245	76	95	4
Overlap of targets with wildtype (no.)		12	6	5	0
Overlap of		4.9%	7.9%	5.3%	0%

targets with wildtype (%)					
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Supplementary Table S3: Comparison of pretreatment characteristics of CN-AML patients according to miR-142-3p expression levels.

Characteristic	High miR-142-3p expressers (n=119)	Low miR-142-3p expressers (n=118)	P
Age, years			1
Median	48	47	
range	19-60	17-60	
Sex			.17
male - no. (%)	67 (56)	56 (47)	
female - no. (%)	52 (44)	62 (53)	
FAB-Subtype			.46
M0 - no. (%)	4 (3)	3 (2)	
M1 - no. (%)	21 (18)	26 (22)	
M2 - no. (%)	23 (19)	29 (25)	
M4 - no. (%)	46 (38)	35 (30)	
M5 - no. (%)	21 (18)	14 (12)	
M6 - no. (%)	1 (1)	5 (4)	
M7 - no. (%)	1 (1)	2 (2)	
missing data - no. (%)	2 (2)	4 (3)	.86
Peripheral blood blasts			
median (%)	53	57	
missing data – no. (%)	4 (3)	6 (5)	1.0
Bone marrow blasts			
median (%)	80	80	
missing data – no. (%)	11 (9)	8 (7)	
Type of AML			.48
de novo - no. (%)	110 (92)	106 (90)	
secondary - no. (%)	9 (8)	12 (10)	
WBC count			.54
median - (x10 ⁹ /l)	24.6	27.85	
range - (x10 ⁹ /l)	0.65–239.4	0.5-328.2	
missing data – no. (%)	0 (0)	0 (0)	
Hemoglobin			.38
median – g/L	9.05	9.05	
range – g/L	5.3-13.5	3-14.9	
missing data – no. (%)	5 (4)	2 (2)	
Platelet count			.9
median - (x10 ⁹ /l)	51	55	

range - (x10 ⁹ /l)	11-332	4-373	
missing data – no. (%)	6 (5)	2 (2)	
ECOG performance status			.14
0 - no. (%)	23 (19)	27 (23)	
1 - no. (%)	86 (72)	72 (61)	
2 - no. (%)	9 (8)	17 (14)	
missing data - no. (%)	1 (1)	2 (2)	
<i>FLT3</i> -ITD – no. (%)			.38
mutated– no. (%)	37 (31)	43 (36)	
Wildtype - no. (%)	81 (68)	74 (63)	
missing – no. (%)	1 (1)	1 (1)	
<i>NPM1</i>			.69
mutated – no. (%)	67 (56)	70 (59)	
Wildtype - no. (%)	50 (42)	47 (40)	
missing – no. (%)	2 (2)	1 (1)	
<i>NPM1</i> mutated/ <i>FLT3</i> -ITD negative			.93
low risk* – no. (%)	41 (34)	40 (34)	
high risk* – no. (%)	77 (65)	77 (65)	
missing – no. (%)	1 (1)	1 (1)	
<i>CEBPA</i>			.71
mutated – no. (%)	13 (11)	15 (13)	
Wildtype - no. (%)	104 (87)	103 (87)	
missing – no. (%)	2 (2)	0 (0)	
<i>DNMT3A</i>			.97
mutated – no. (%)	34 (29)	34 (29)	
Wildtype - no. (%)	85 (71)	84 (71)	
missing – no. (%)	0 (0)	0 (0)	
<i>WT1</i>			.86
mutated – no. (%)	15 (13)	14 (12)	
Wildtype - no. (%)	104 (87)	104 (88)	
missing – no. (%)	0 (0)	0 (0)	
<i>NRAS</i>			.057
mutated – no. (%)	20 (17)	10 (8)	
Wildtype - no. (%)	97 (81)	105 (89)	
missing – no. (%)	2 (2)	3 (3)	
<i>IDH1</i>			.65
mutated – no. (%)	11(9)	13 (11)	
Wildtype - no. (%)	108 (91)	105 (89)	
missing – no. (%)	0 (0)	0 (0)	
<i>IDH2</i>			.69
mutated – no. (%)	13 (11)	15 (13)	
Wildtype - no. (%)	105 (88)	103 (87)	
missing – no. (%)	1 (1)	0 (0)	

Abbreviations: AML, acute myeloid leukemia; FAB, French-American-British classification of acute myeloid leukemia; WBC, white blood cell count; ECOG, performance status of the Eastern Cooperative Oncology Group; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; P, p-value from two-sided Chi-squared tests for categorical variables and from two-sided Mann-Whitney-U tests for continuous variables; * The high-risk molecular group is defined as either *NPM1*^{wildtype}/*FLT3*-ITD^{negative}, or *NPM1*^{wildtype}/*FLT3*-ITD^{positive}, or *NPM1*^{mutated}/*FLT3*-ITD^{positive}. The low-risk molecular group is defined by the presence of an *NPM1* mutation and the absence of *FLT3*-ITD.

Supplementary Table S4: Comparison of pretreatment characteristics of CN-AML patients according to miR-142-5p expression levels.

Characteristic	High miR-142-5p expressers (n=119)	Low miR-142-5p expressers (n=118)	P
Age, years			.93
Median	48	47	
range	19-60	17-60	
Sex			.4
male - no. (%)	65 (55)	58 (49)	
female - no. (%)	54 (45)	60 (51)	
FAB-Subtype			.86
M0 - no. (%)	4 (3)	3 (3)	
M1 - no. (%)	20 (17)	27 (23)	
M2 - no. (%)	25 (21)	27 (23)	
M4 - no. (%)	44 (37)	37 (31)	
M5 - no. (%)	20 (17)	15 (12)	
M6 - no. (%)	3 (2)	3 (3)	
M7 - no. (%)	1 (1)	2 (2)	
missing data - no. (%)	2 (2)	4 (3)	
Peripheral blood blasts			.44

median (%)	48	59	
missing data – no. (%)	4 (3)	6 (5)	
Bone marrow blasts			.65
median (%)	80	80	
missing data – no. (%)	11 (9)	8 (7)	
Type of AML			.8
de novo - no. (%)	109 (92)	107 (91)	
secondary - no. (%)	10 (8)	11 (9)	
WBC count			.75
median - (x10 ⁹ /l)	23.2	27.85	
range - (x10 ⁹ /l)	.65-328.2	.5-262	
missing data – no. (%)	0 (0)	0 (0)	
Hemoglobin			.87
median – g/L	9	9.2	
range – g/L	5.3-13.5	3-14.9	
missing data – no. (%)	4 (3)	3 (3)	
Platelet count			.95
median - (x10 ⁹ /l)	50.5	55	
range - (x10 ⁹ /l)	7-332	4-373	
missing data – no. (%)	5 (4)	3 (3)	
ECOG performance status			.8
0 - no. (%)	24 (20)	26 (22)	
1 - no. (%)	82 (69)	76 (64)	
2 - no. (%)	12 (10)	14 (12)	
missing data - no. (%)	1 (1)	2 (2)	
FLT3-ITD – no. (%)			.75
mutated– no. (%)	39 (33)	41 (35)	
Wildtype - no. (%)	79 (66)	76 (64)	
missing – no. (%)	1 (1)	1 (1)	
NPM1			.14
mutated – no. (%)	63 (53)	74 (63)	
Wildtype - no. (%)	54 (45)	43 (36)	
missing – no. (%)	2 (2)	1 (1)	
NPM1mutated/FLT3-ITD negative			.2
low risk* – no. (%)	36 (30)	45 (38)	
high risk* – no. (%)	82 (69)	72 (61)	
missing – no. (%)	1 (1)	1 (1)	
CEBPA			.44
mutated – no. (%)	12 (10)	16 (14)	
Wildtype - no. (%)	105 (88)	102 (86)	
missing – no. (%)	2 (2)	0 (0)	
DNMT3A			.41
mutated – no. (%)	37 (31)	31 (26)	

Wildtype - no. (%)	82 (69)	87 (74)	
missing – no. (%)	0 (0)	0 (0)	
<i>WT1</i>			.82
mutated – no. (%)	14 (12)	15 (13)	
Wildtype - no. (%)	105 (88)	103 (87)	
missing – no. (%)	0 (0)	0 (0)	
<i>NRAS</i>			.28
mutated – no. (%)	18 (15)	12 (10)	
Wildtype - no. (%)	100 (84)	102 (86)	
missing – no. (%)	1 (1)	4 (3)	
<i>IDH1</i>			.38
mutated – no. (%)	10 (8)	14 (12)	
Wildtype - no. (%)	109 (92)	104 (88)	
missing – no. (%)	0 (0)	0 (0)	
<i>IDH2</i>			.69
mutated – no. (%)	15 (12)	13 (11)	
Wildtype - no. (%)	103 (87)	105 (89)	
missing – no. (%)	1 (1)	0 (0)	

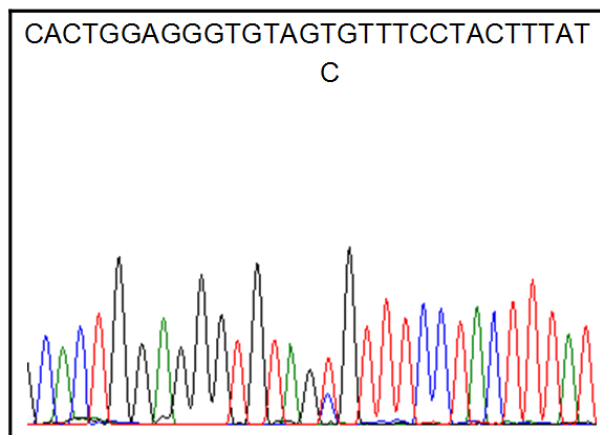
Abbreviations: AML, acute myeloid leukemia; FAB, French-American-British classification of acute myeloid leukemia; WBC, white blood cell count; ECOG, performance status of the Eastern Cooperative Oncology Group; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; P, p-value from two-sided Chi-squared tests for categorical variables and from two-sided Mann-Whitney-U tests for continuous variables; * The high-risk molecular group is defined as *either* $NPM1^{wildtype}/FLT3-ITD^{negative}$, or $NPM1^{wildtype}/FLT3-ITD^{positive}$, or $NPM1^{mutated}/FLT3-ITD^{positive}$. The low-risk molecular group is defined by the presence of an *NPM1* mutation and the absence of *FLT3*-ITD.

Supplemental Figures

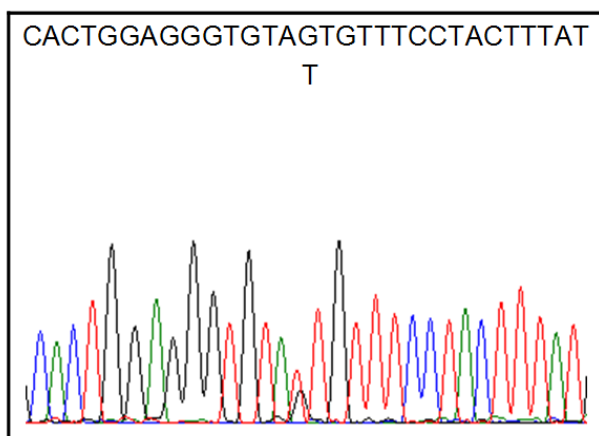
Supplementary Figure S1. Sequence traces from patients with mutated microRNAs.

Nucleotide sequence numbering is according to the coding DNA sequence of UCSC Genome Browser (Genomes March 2006, NCBI 36/hg18)

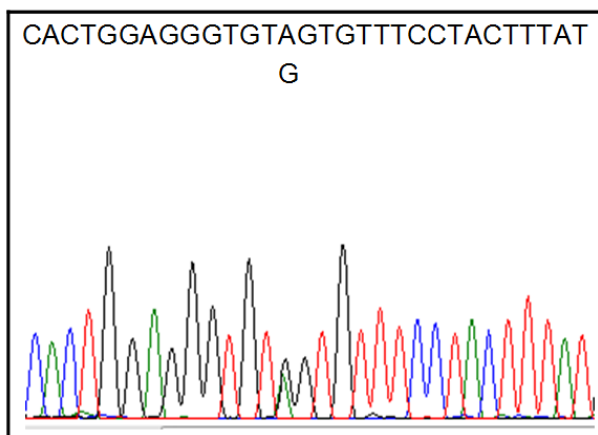
Patient 1: miR-142-3p: Chr.17:53763622 T>C , abbreviation : pos54T>C



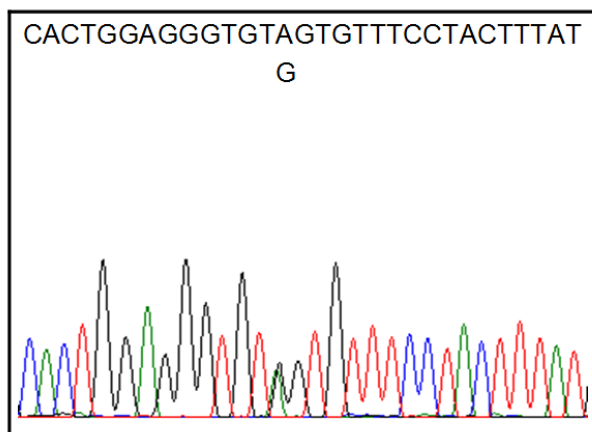
Patient 2: miR-142-3p: Chr.17:53763623 G>T, abbreviation: pos55G>T



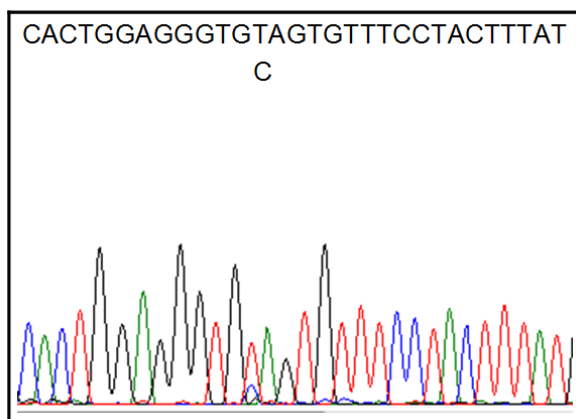
Patient 3: miR-142-3p: Chr.17: 53763624 A>G, abbreviation : pos56A>G



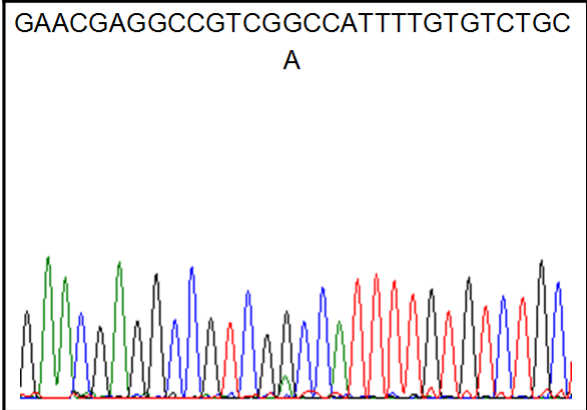
Patient 4: miR-142-3p: Chr.17: 53763624 A>G, abbreviation: pos56A>G



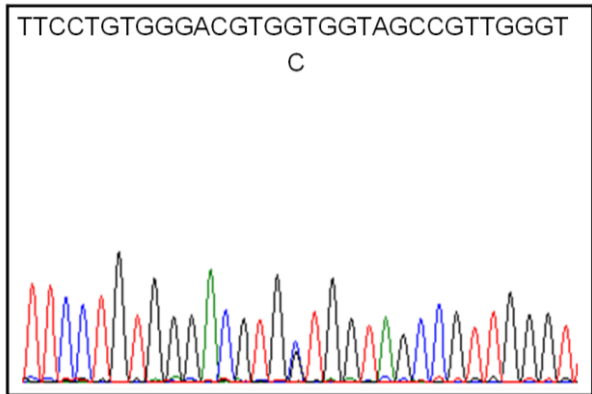
Patient 5: miR-142-3p: Chr.17:53763625 T>C, abbreviation: pos57T>C



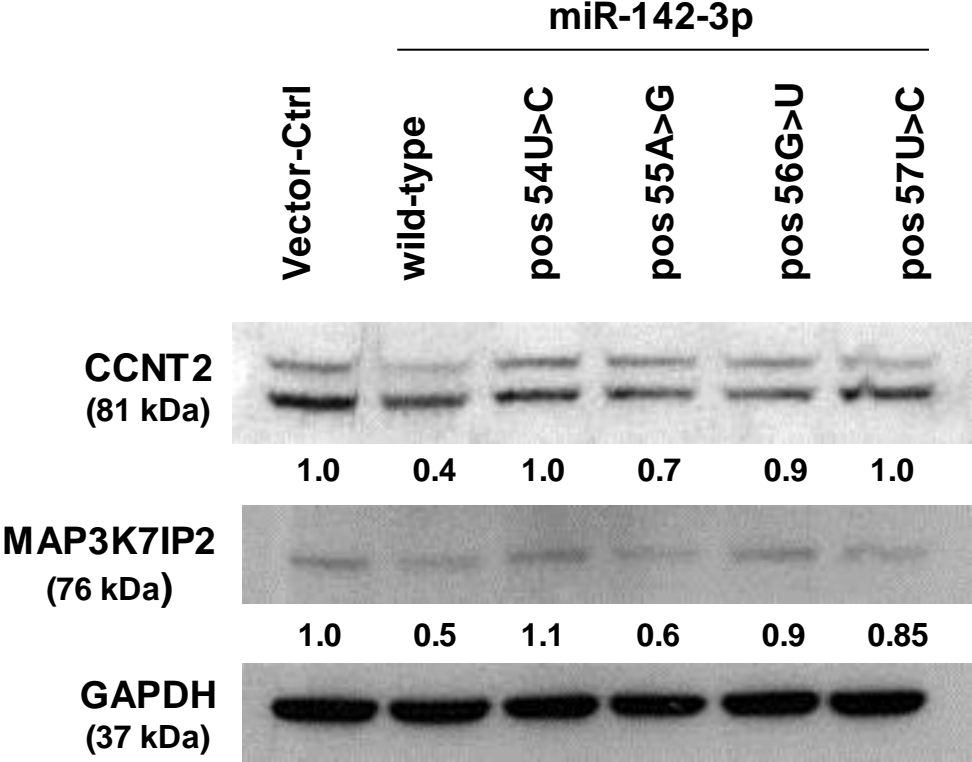
Patient 6: miR-632:Chr.17:27701293 G>A



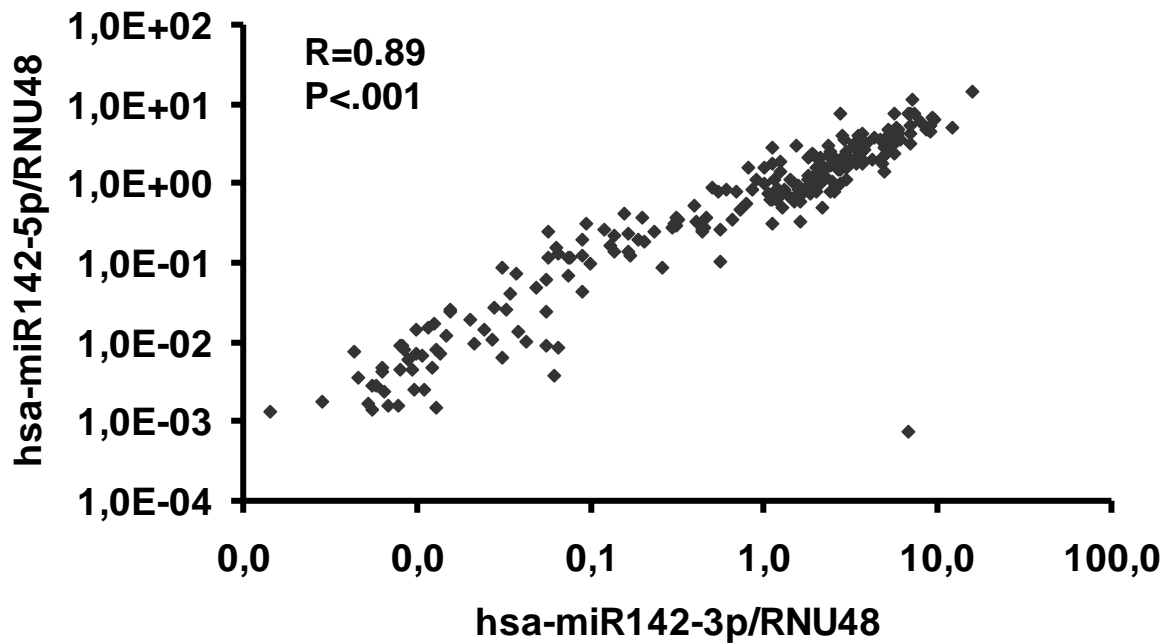
Patient 7: miR-632:Chr.17: 27701324 G>C



Supplementary Figure S2. Western blot of miR-142-3p target genes CCNT2 and MAP3K7IP2 in 293T cells transduced with CTRL or miR-142-3p wildtype or mutated vectors as indicated.



Supplementary Figure S3. Correlation of miR-142-3p and miR-142-5p transcript levels in CN-AML patients.



Supplementary Reference

1. Scherr M, Battmer K, Ganser A, Eder M. Modulation of gene expression by lentiviral-mediated delivery of small interfering RNA. *Cell Cycle*. 2003 May-Jun;2(3):251-7.