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

MicroRNAs are short (20-40 nucleotides) non-coding RNA molecules that are responsible for the post-transcriptional regulation of gene expression. Aberrant expression of microRNAs has been associated with various malignancies.[1,2] Specifically, downregulation of microRNA-142 (miR-142) has been shown to occur in acute myeloid leukemia (AML).[3] Interestingly, also gene mutations in miR-142, miR-632 and miR-891 have been recently described in *de novo* AML.[4] So far, little is known about mutations in miR-142 and other related microRNAs in myeloid malignancies. The aim of this study was to analyze mutations in the miR-142 and related microRNAs, such as miR-632, and miR-891, in a large cohort of 935 patients with AML or myelodysplastic syndrome (MDS).

The patient group consisted of 416 *de novo* AML patients (excluding AML M3) who entered the multicenter treatment trials AMLSHG-0199 or AMLSHG-0295, 326 patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) for secondary AML after a prior diagnosis of MDS (sAML) (n=170) or primary MDS (n=156), and 193 primary MDS patients not undergoing intensive therapy or allogeneic HSCT. Informed consent was obtained in accordance with the Declaration of Helsinki. This study was approved by the institutional review board of Hannover Medical School.

The genomic region of the miR-142 gene (containing miR-142-5p and miR-142-3p) (all patients), miR-632 (CN-AML and MDS patients) and miR-891 (CN-AML and MDS patients) were sequenced by Sanger sequencing as previously described[5] and with primers listed in *Online Supplementary Table S1*. Patients' samples were also assessed for other frequently mutated genes in AML and MDS, as previously described (*Online Supplementary Appendix*).[6-9] All mutations were confirmed in an independent experiment. Sequence analysis was performed with the Mutation Surveyor software (Softgenetics, State College, PA, USA). The somatic or germ-line status of mutations in miR-142 was established by evaluating T cells ($CD3^+CD11b^-CD14^-CD33^-$) purified from diagnostic samples by flow cytometry when available (n=2).

Gene expression levels of miR-142-3p and miR-142-5p were assessed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) as detailed in the *Online Supplementary Appendix*. Overall survival (OS), complete remission (CR) and relapse-free survival (RFS) were calculated as previously described.[10,11] The statistical analyses were performed with the statistical software package SPSS 21.0 (IBM Corporation, Armonk, NY, USA).

In the whole cohort, we identified 7 patients with mutations in miRNAs (0.74%) (Table 1). All mutations were heterozygous (*Online Supplementary Figure S1*). MiR-142-3p was the most frequently affected miRNA with 5 patients showing a mutation (0.53%) (Figure 1A and *Online Supplementary Figure S1*). The somatic origin could be confirmed for miR-142-3p by analyzing T cells which did not show the mutation (n=2). All of these mutations affected the seed region of miR-142-3p, thereby potentially changing the target specificity of miR-142. In line with this, target prediction for miR-142-3p with targetscan (release 5.2: June 2011; available from: *www.targetscan.org/vert_50/*) showed 250 conserved targets, while three of four mutations had much fewer predicted targets [pos54U>C: 245 conserved targets, pos55A>G: 76, pos56G>U: 95, and pos57U>C: 4 conserved targets (*Online Supplementary Table S2*)]. The overlap of the predicted target genes was 0 to 7.9% between wild-type and mutated miR-142-3p in these four positions, suggesting that mutations in miR-142 are loss-of-function mutations. Functional validation of the mutated miRNAs confirmed that mutations of miR-142-3p pos54U>C, pos56G>U, and pos57U>C had little or no effect on protein expression of miR-142-3p targets CCNT2 and MAP3K7IP2 (TAB2)[12] in leukemic and fibroblast cells in contrast to wild-type miR-142-3p, which knocked down protein expression (Figure 1B and *Online Supplementary Figure S2*). The partial knockdown of target genes by the mutation at pos55A>G can be explained by the wobble effect of guanosine.

Mutations in miR-142 occurred in male and female patients (Table 1). Of the 5 patients with mutations in miR-

Table 1. Characteristics of miR-142 and miR-632 mutated patients.

Patient	Age (years)	Sex	WHO/ FAB	IPSS	Transplant	Karyotype	Mutated miRNA	Nucleotide change	sAML	Survival (years), status	Other mutations
1	42	F	RA	n.d	n.d.	n.d.	miR-142	Chr.17: 53763622 T>C	n.d.	n.d.	n.d.
2	57	M	AML	n.a.	no	46,XY	miR-142	Chr.17:53763623 G>T	n.a.	0.24, dead	none
3	69	M	RA	Int-1	no	46,XY	miR-142	Chr.17: 53763624 A>G	yes	0.5, dead	SRSF2
4	61	F	sAML	n.a.	yes	46,XX	miR-142	Chr.17: 53763624 A>G	n.a.	0.58, dead	U2AF1
5	59	F	sAML	n.a.	yes	46,XX	miR-142	Chr.17: 53763625 T>C	n.a.	2.73, alive[#]	IDH1 nRAS
6	57	F	AML M1	n.a.	no	46,XX	miR-632	Chr.17: 27701293 G>A	n.a.	0.50, dead	IDH2, DNMT3A
7	73	M	RA	n.d	n.d.	n.d.	miR-632	Chr.17: 27701324 G>C	n.d.	n.d	n.d.

F: female; M: male; sAML: secondary acute myeloid leukemia after a prior diagnosis of myelodysplastic syndrome; RA: refractory anemia; n.a.: not applicable; n.d.: no data available. [#]Patient underwent allogeneic stem cell transplantation.

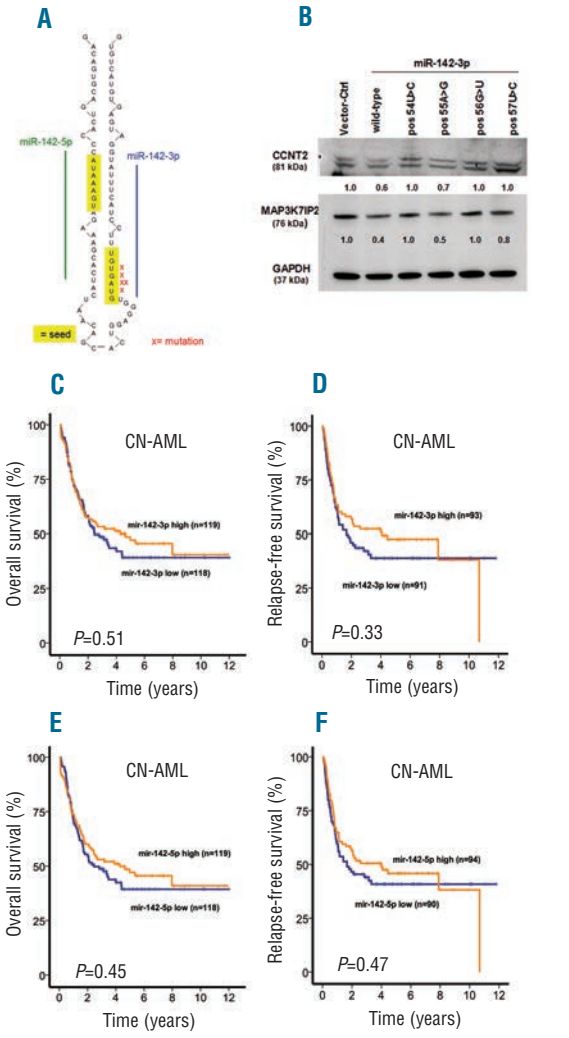

Figure 1. (A) Location of mutations in miR-142 in patients with AML and MDS. (B) Western Blot of miR-142-3p target genes CCNT2 and MAP3K7IP2 in THP1 cells transduced with CTL or miR-142-3p wild-type or mutated vectors as indicated. (C) Overall and (D) relapse-free survival of patients with CN-AML according to miR-142-3p expression levels above or below the median expression (log rank test). (E) Overall and (F) relapse-free survival of patients with CN-AML according to miR-142-5p expression levels above or below the median expression (log rank test).

142, only one patient carried the diagnosis of *de novo* AML (0.2% in *de novo* AML), while 2 patients were diagnosed with sAML (1.2% in sAML) and 2 patients had MDS (0.56% in MDS, corresponding to 0.77% in MDS/sAML from MDS). Apart from one patient who underwent allogeneic transplantation for sAML and was alive 2.73 years later, all other patients with follow-up data died of the disease in less than one year. Four patients had normal cytogenetics; while for one patient cytogenetic information was not available. Myelodysplasia-related gene mutations, such as mutations in the splicing genes or chromatin remodeling genes, were found in 2 patients (one patient with mutated *ASXL1* and *SRSF2*, one patient with mutated *U2AF1*). One patient had a concomitant mutation in *NRAS* and *IDH1* (Table 1). The associated mutational profile could possibly suggest that miR-142 mutations play a role in the pathogenesis of MDS rather than *de novo* AML. Two patients were identified with heterozygous missense mutations in miR-632 (Table 1). One of these patients was a female AML patient with a normal karyotype but with concomitant mutations in *IDH2* and *DNMT3A*; the patient died six months after diagnosis. The other miR-632 mutated patient carried the diagnosis of MDS and was classified as refractory anemia (RA) (Table 1). While the 2 patients had different nucleotides of miR-632 affected by the mutations, neither variant affected the seed region. Interestingly, miR-632 expression was found to be lower in MDS patients as compared to normal controls in a prior study[13] suggesting that a functional loss or reduction of miR-632 could be involved in the pathogenesis of MDS. None of the evaluated patients carried a mutation in miRNA-891A suggesting that this miRNA is only very rarely mutated in myeloid malignancies.

Because of data suggesting that aberrant miR-142-3p expression is associated with myeloid malignancies,[14,15] we also evaluated the expression of miR-142-3p and miR-142-5p in 237 patients with CN-AML. Relative miR-142-3p and miR-142-5p transcript levels ranged from 0.0014 to 15.80 and from 0 to 15.14 of *miRNA*/RNU48 copies in CN-AML patients, respectively. The relative transcript levels of miR-142-3p and miR-142-5p in the miR-142 mutated CN-AML patients were 1.79 and 1.16, respectively. Relative miR-142-3p and miR-142-5p transcript levels were highly correlated (R=0.89, $P<0.001$) (*Online Supplementary Figure S3*).

We found that patients with levels above or below the median miR-142-3p expression showed no difference in OS (HR 0.89, 95%CI: 0.63-1.26; $P=0.51$), RFS (HR 0.82, 95%CI 0.56-1.22; $P=0.033$) and CR (78% *vs.* 77%; $P=0.85$) (Figure 1C and D). Similar results were obtained for miR-142-5p with expression above or below the median not affecting OS (HR 0.88, 95%CI: 0.62-1.24; $P=0.45$), RFS (HR 0.87, 95%CI: 0.59-1.28; $P=0.47$) and CR (79% *vs.* 76%; $P=0.62$) (Figure 1E and F). CN-AML patients with high *versus* low expression of miR-142-3p and miR-142-5p showed no significant difference in clinical parameters such as age, sex, FAB subtype, blast count, type of AML, ECOG performance status as well as in mutation frequencies of *FLT3-ITD, NPM1, CEBPA, DNMT3A, WT1, NRAS, IDH1* and IDH2 (*Online Supplementary Tables S3 and S4*). Only a trend towards a higher *NRAS* mutation rate could be observed in the miR-142-3p high *versus* low expressing group (17 *vs.* 8.5%; $P=0.057$).

In summary, miR-142 is recurrently but infrequently mutated in MDS, *de novo* and secondary AML evolving from MDS. Mutations in miR-632 and miR-891 are even rarer. MicroRNA mutations co-occur with MDS-related gene aberrations like *IDH* mutations and are associated with normal cytogenetics and poor survival. We show that miR-142-3p mutations affecting the seed region of the miRNA reduce target specificity. These data suggest that loss of target control by miR-142 may be a novel mechanism in the pathogenesis of 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 Hofmann,[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 Heuser[1]*

**These authors contributed equally*

[1]Department of Hematology, Hemostasis, Oncology and HSCT, Hannover Medical School, Hannover; [2]Medizinische Klinik und Poliklinik I, Universitätsklinikum Carl Gustav Carus, Dresden;

[3]Klinik für Hämatologie, Onkologie und Klinische Immunologie, Heinreich Heine Universität, Düsseldorf; [4]Department of Medicine, Hematology/Oncology, University Hospital, Frankfurt; [5]Department of Hematology and Oncology, University Hospital Mannheim; [6]Department of Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, Hamburg; [7]Department of Medicine II, Oncological Center, Hubertus Wald University Cancer Center, University Hospital Hamburg-Eppendorf, Hamburg; [8]Department of Internal Medicine III, University of Ulm; and [9]Klinikum Braunschweig, Germany

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Correspondence: thol.felicitas@mh-hannover.de
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