Rare occurrence of *DNMT3A* mutations in myelodysplastic syndromes

Felicitas Thol, Claudia Winschel, Andrea Lüdeking, Haiyang Yun, Inna Friesen, Frederik Damm, Katharina Wagner, Jürgen Krauter, Michael Heuser,* and Arnold Ganser*

Department of Hematology, Hemostasis, Oncology, and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany

ABSTRACT

Gene mutations and epigenetic changes have been shown to play significant roles in the pathogenesis of myelodysplastic syndromes. Recently, mutations in *DNMT3A* were identified in 22.1% of patients with acute myeloid leukemia. In this study, we analyzed the frequency and clinical impact of *DNMT3A* mutations in a cohort of 193 patients with myelodysplastic syndromes. Mutations in *DNMT3A* were found in 2.6% of patients. The majority of mutations were heterozygous missense mutations affecting codon R882. Patients with *DNMT3A* mutations were found to have a higher rate of transformation to acute myeloid leukemia. When assessing the global methylation levels in patients with mutated *versus* unmutated *DNMT3A* and healthy controls no difference in global DNA methylation levels between the two groups was seen. Our data show that in patients with myelodysplastic syndromes, *DNMT3A* mutations occur at a low frequency and may be a risk factor for leukemia progression.

Key words: DNMT3A mutations, MDS occurrence.

Citation: Thol F, Winschel C, Lüdeking A, Yun H, Friesen I, Damm F, Wagner K, Krauter J, Heuser M, and Ganser A. Rare occurrence of DNMT3A *mutations in myelodysplastic syndromes. Haematologica 2011;96(12):1870-1873. doi:10.3324/haematol.2011.045559*

Introduction

Myelodysplastic syndromes (MDS) are heterogeneous disorders of the hematopoietic stem cell caused by mutations, deregulated gene expression, and epigenetic modifications of genes leading to inefficient hematopoiesis and a propensity to transform to acute myeloid leukemia (AML). The aim of the present study is to characterize the frequency and clinical impact of a recently identified mutation in a key epigenetic regulator: *DNA methyltransferase 3A* (*DNMT3A*). DNA methylation has been shown to affect prognosis[1] and treatment response in MDS,[2] and demethylating agents have recently been introduced successfully into the management of the disease.[3] Methyltransferases, such as DNMT1, DNMT3A and DNMT3B, are important for epigenetic regulation of genes as they catalyze the addition of methyl groups to the cytosine residue of CpG dinucleotides. While DNMT3A and DNMT3B are important for *de novo* methylation, DNMT1 is essential for methylation maintenance. Functional studies evaluating the significance of these genes at the hematopoietic stem cell level have suggested that DNMT1 is important for self-renewal of hematopoietic stem cells.[4] Interestingly, somatic mutations in *DNMT3A* have been recently described in 62 out of 281 (22.1%) patients with AML[5] with the highest frequency (33.7%) being found in patients with cytogenetically normal (CN-) AML. In this study, 18 different *DNMT3A* mutations were described in AML with the majority being missense mutations and a smaller number of nonsense and frameshift mutations.[5] The most common missense mutation affecting codon R882 was found in 37 out of 62 mutated patients (59.7%). Ley *et al.* described an adverse prognostic impact of the mutation for patients in AML.[5] Less is known about the frequency and the prognostic impact of *DNMT3A* mutations in MDS. However, one study suggests that mutations are less frequent than in AML.[6] Here, we report frequency and clinical impact of mutations of *DNMT3A* in a large cohort of 193 MDS patients. Characteristics regarding *IDH1/2*, *NPM1*, and *ASXL1* mutations for patients in this cohort have been previously reported.[7,8]

Design and Methods

Cell samples from 193 MDS patients were collected on enrolment in clinical trials. Patients were enrolled in multicenter treatment trials that investigated the use of antithymocyte globulin[9] (*ClinicalTrials Identifier NCT00004208*)[10], ATRA,[11] deferasirox,[12] lenalidomide, or thalidomide for treatment of MDS while demethylating agents were not used in this patient cohort. DNA from 80 healthy blood donors (age 18-60 years) was obtained from the Institute of Transfusion Medicine, Hannover Medical School, Germany.

**Denotes equal contribution*

Acknowledgments: we are indebted to all patients and contributing doctors, especially Dr G Bug, PD, Dr Oliver Ottmann and Prof. Dr W-K Hofmann. We thank Kerstin Görlich and Martin Wichmann for their excellent support in sample and data acquisition. We thank Prof. R Blasczyk from the Department of Transfusion Medicine at Hannover Medical School for the supply of healthy control samples.

Funding: this study was supported by the Dieter-Schlag Stiftung, grant N. DJCLS R 10/22 from the Deutsche-José-Carreras Leukämie-Stiftung e.V., grant N. 109003 and 109686 from the Deutsche Krebshilfe e.V, and grant N. M 47.1 from the H. W. & J. Hector Stiftung.

Manuscript received on April 6, 2011. Revised version arrived on August 15, 2011. Manuscript accepted on August 18, 2011.

Correspondence: Felicitas Thol, Department of Hematology, Hemostasis, Oncology, and Stem Cell Transplantation, Hannover Medical School, Carl-Neuberg Str. 1, 30625 Hannover, Germany. Phone: international +49.511.5329782. Fax: international +49.511.5329783. E-mail thol.felicitas@mh-hannover.de

Clinical and hematologic data were recorded after MDS patients gave their informed consent in accordance with the Declaration of Helsinki, and the scientific analysis of the samples was approved by the institutional review board of Hannover Medical School (ethical approval N. 2467). Among 193 MDS patients, follow-up information was available for 154 patients. The follow-up information was updated by means of clinic visits as well as telephone calls to patients, their doctors, or local registration offices. According to the WHO classification, our cohort included patients with refractory anemia (RA; n=38), refractory anemia with ringed sideroblasts (RARS; n=20), MDS with isolated del(5q) (del5q; n=18), refractory cytopenia with multilineage dysplasia (RCMD; n=30); refractory anemia with excess blasts-1 (RAEB-1; n=22), refractory anemia with excess blasts-2 (RAEB-2; n=31) and MDS-unclassifiable (MDS-U; n=7). Twenty-seven patients had no WHO-subtype information available. IPSS was low in 39, intermediate-1 in 57, intermediate-2 in 38, and high in 13 patients (46 patients had no IPSS information available).

First, all 23 exons of *DNMT3A* were analyzed for mutations in 40 patients (20 AML and 20 MDS patients). The AML patients were entered into the multicenter treatment trials AML SHG 0199 (*ClinicalTrials Identifier NCT00209833*, June 1999 to September 2004) or AML SHG 0295 (February 1995 to May 1999). Since we only found mutations between exons 15-23 in the 40 AML and MDS patients, for the remaining 173 MDS patients we subsequently amplified the genomic regions of exons 15-23 of *DNMT3A* as previously reported.[13] PCR fragments were directly sequenced, and were analyzed using the Sequencing Analysis 5.3.1 software (Applied Biosystems, Darmstadt, Germany) and Vector NTI Advance 10 software (Invitrogen, Karlsruhe, Germany). All mutations were confirmed in an independent experiment.

Global methylation of CpG islands was assessed in duplicate using the Imprint Methylated DNA Quantification Kit (Sigma-Aldrich) in bone marrow samples from *DNMT3A* mutated (n=3) or wild-type MDS patients (n=20), and in peripheral blood samples from healthy volunteers (n=10). Global DNA methylation is shown as percent methylation of a methylated control DNA which was provided by the manufacturer. For gene expression analysis total RNA was isolated using the All Prep DNA/RNA Kit (Qiagen). Random hexamer priming and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) were used to generate cDNA. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out on a StepOne Plus real-time PCR system (Applied Biosystems) using the *DNMT3A* gene expression assay (Applied Biosystems, Assay ID *DNMT3A*: Hs01027166_m1). cDNA from the KG1A cell line was used to construct a standard curve for *DNMT3A*. *ABL* (*ABL* Fusion*Quant* Standards; Ipsogen, Marseille, France) was quantified as a control gene. Patients were divided into two groups at the median level of *DNMT3A/ABL* expression. The two-sided level of significance was set at $P<0.05$. The statistical analyses were performed with the SPSS Version 18 software package.

Results and Discussion

Somatic mutations in *DNMT3A* were present in 5 patients (2.6%) with the majority of mutations being heterozygous missense mutations affecting codon R882 (n=3) (Figure 1). A nonsense mutation in the zinc finger domain was found in one patient and a frameshift mutation was found in the methyltransferase domain of another patient (Figure 1). Sequencing of exon 23 in 80 healthy volunteers did not identify any mutation. The low frequency of *DNMT3A* mutations in MDS did not allow any formal assessment of clinical and molecular associations or prognostic evaluation. However, when looking at clinical characteristics of the mutated patients, we found mutations in patients with different WHO classifications, karyotypes and IPSS scores, suggesting that the mutation can occur in different cytogenetic and clinical groups of MDS. Ley *et al.* described an association between *IDH1* and *NPM1* in AML. Probably also due to the low number of mutations in *DNMT3A*, *IDH* and *NPM1* found in our MDS cohort, we could not find such an association in MDS (Table 1). One patient had a concurrent mutation in *ASXL1*, and one in *IDH1*, while the other 3 patients had no mutation in *IDH* or *ASXL1*. Three of the 4 mutated patients with available follow-up information developed a secondary AML (75%) compared to 28.2% of patients with wild-type *DNMT3A* (P=0.043): AML transformation rate in the whole cohort 29.4%. The mutated patient with follow-up information who did not develop AML underwent allogeneic stem cell transplantation approximately seven

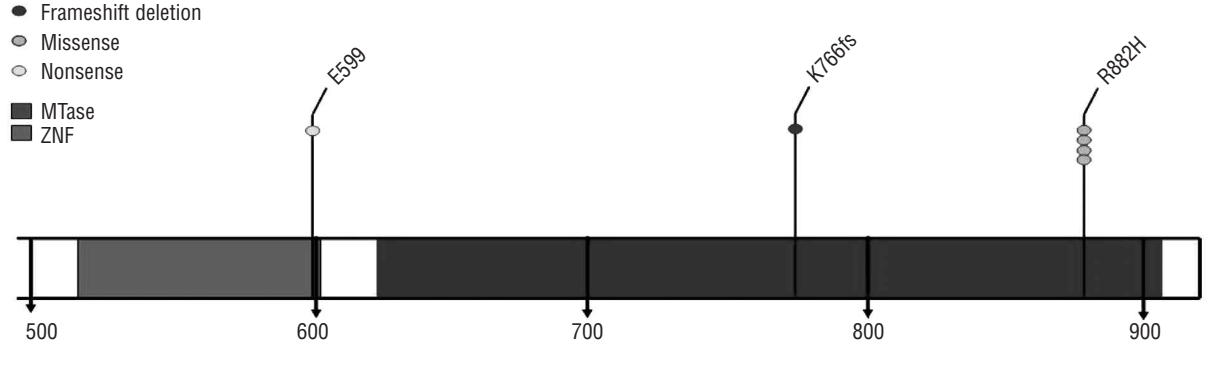

Figure 1. Location and type of *DNMT3A* mutations in MDS patients. Genomic DNA from 193 MDS patients was extracted from bone marrow or peripheral blood at time of enrolment in clinical trials, and exons 15 to 23 of *DNMT3A* (Ensembl gene ENSG00000119772) containing the ZNF and the methyltransferase domain were sequenced by sanger sequencing. Five mutations with translational effects were identified (2.6 %). Each patient with a *DNMT3A* mutation is designated with a circle: ZNF, FYVE/PHD-type zinc finger domain; MTase: methyltransferase domain (MTase); E: glutamic acid; K: lysine; fs: frameshift mutation; R: arginine; H: histidine.

months after initial diagnosis and has since been in remission. The other 3 patients with the mutation and available follow-up information had a very short median overall survival (0.6 years), while the median survival of the patients without mutated *DNMT3A* was 3.04 years. *DNMT3A* influences epigenetic regulation of genes by adding methyl groups to the cytosine residue of CpG dinucleotides. We, therefore, also assessed the global methylation levels in MDS patients with mutated *DNMT3A versus* unmutated *DNMT3A* and healthy controls. No differences were found in global methylation levels between mutated and wild-type patients (Figure 2) as already described by Ley *et al.* in AML, while Yan *et al.* identified a decrease in methylation of CpG islands in the *HOXB* cluster which may suggest an activation of stem cell self-renewal pathways in the mutated patients.[5,14] Patients with mutated *DNMT3A* showed lower *DNMT3A* expression levels (relative copy number *DNMT3A/ABL* 9.22×10^{-5}; mean n=5) compared to patients with wild-type *DNMT3A* (relative copy number *DNMT3A/ABL* 4.37×10^{-4}; mean n=163). However, this difference was not significant (P=0.4). There were no significant differences in patients with low (n=79) *versus* high (n=79) *DNMT3A* expression for overall survival (P=0.39) and time to AML transforma-

Table 1. ***DNMT3A*** **mutations in patients with MDS.**

Patient	Age	Sex	WHO	IPSS	Transfusion dependence	Karyotype	Mutation	sAML (years to AML)	Survival (years), status#	Other mutations
1	59	F	RAEB-2	Int-2	Yes	46,XX	E599*	Yes (0.6)	0.60 (d)	ASXL1 WT IDH1 mutated NPM1 WT
2	70	F	del(5q)	Int-1	Yes	46,XX,del(5)(q?14q?34)	R882H	Yes (0.03)	0.11 (d)	ASXL1 WT IDH WT NPM1 WT
3	68	F	RAEB-1	Int-2	Yes	46,XX	R882H	Yes (0.55)	1.19 (d)	ASXL1 mutated IDH WT NPM1 WT
4	53	M	RCMD	Int-1	Yes	47,XY,+8	K766fs	No	3.79 (a) Transplanted[§]	ASXL1 WT IDH WT NPM1 WT
5	64	M	RARS	Low	Unknown	46,XY	R882H	Unknown	Unknown	ASXL1 WT IDH WT NPM1 WT

*F: female; M: male; RARS: refractory anemia with ringed sideroblasts; del5q: MDS with isolated del(5q); RCMD: refractory cytopenia with multilineage dysplasia; RAEB-1: refractory anemia with excess blasts-1; RAEB-2: refractory anemia with excess blasts-2; IPSS: International Prognostic Scoring System; int-1: intermediated 1; int-2: intermediated 2, sAML: secondary acute myeloid leukemia after a prior diagnosis of myelodysplastic syndrome; E: glutamic acid; *: stop codon; r: arginine; h: histidine; k: lysine; fs: frameshift mutation; # survival status; d: dead; a: alive; §transplanted: allogeneic stem cell transplantation; WT: wild type.*

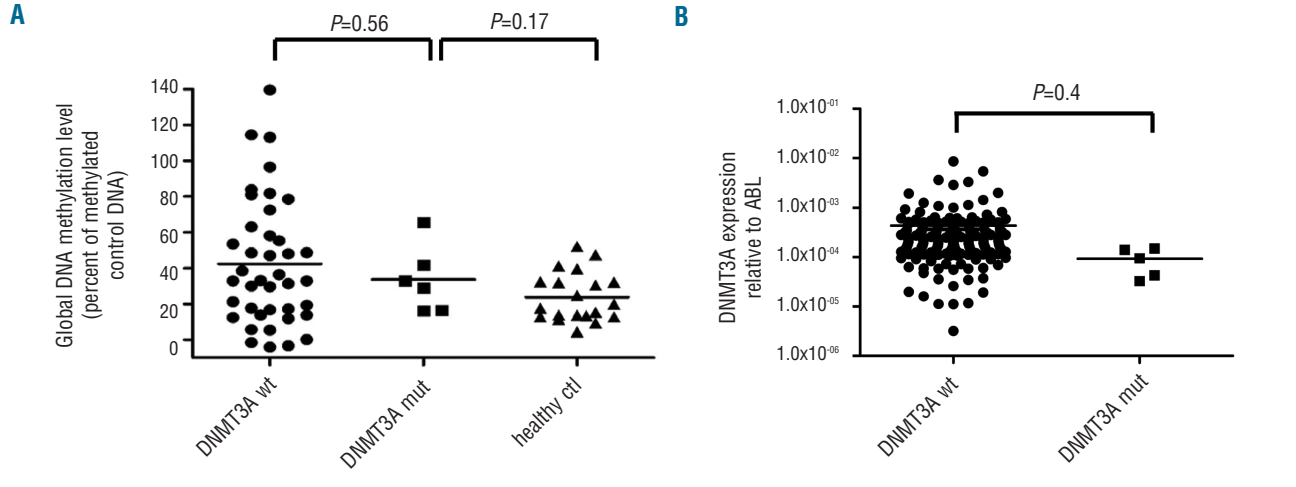

Figure 2. Global DNA methylation levels and DNMT3A expression levels in DNMT3A mutated and wild-type MDS patients and healthy controls. (A) Global methylation of CpG islands was assessed using the Imprint Methylated DNA Quantification Kit (Sigma-Aldrich) in bone marrow samples from randomly selected *DNMT3A* mutated (n=3, in duplicate) or wild-type MDS (n=20, in duplicate) patients, and in peripheral blood samples from healthy volunteers (n=10, in duplicate). Global DNA methylation is shown as percent methylation of a methylated control DNA which was provided by the manufacturer. (B) *DNMT3A* expression levels quantified by RT-PCR and analyzed with graph pad prism 5 (GraphPad Software, La Jolla, USA) comparing patients with wild-type (n=163) and mutated *DNMT3A* (n=5).

tion (P=0.81). Our data demonstrate that *DNMT3A* mutations are present in myeloid malignancies other than AML. However, in MDS the frequency of the mutation is significantly lower than in AML suggesting that mutations in *DNMT3A*, similar to mutations in *IDH1/2*,[7,15,16] are much more prevalent in AML than in MDS. Like AML, the majority of mutations (60%) were heterozygous point mutations located in codon R882. Due to the low frequency of the mutation in MDS, formal prognostic evaluation could not be performed, but our data suggest a possible negative prognostic impact, as already described for mutations in AML. In our cohort, mutated patients more frequently progressed to AML and had a shorter overall survival. Thus, *DNMT3A* mutations might be involved in the progression of the disease. We can, therefore, confirm data from Walter *et al.* who also found that patients with mutated *DNMT3A* had a shorter overall survival time and a more rapid progression to AML compared to patients without the mutation.[6] The mutation rate of 2.6% in our MDS cohort is lower than the recently reported mutation rate of 8% by Walter *et al.* in his MDS cohort.[6] As MDS is a very heterogeneous disease, study cohorts are also likely to be heterogeneous. It is not, therefore, surprising that our cohort included more patients with low risk MDS (i.e. without excess of blasts, low/int-1 risk IPSS) than the cohort reported by Walter *et al.*[6] The latter study also included a few patients with secondary AML (RAEB-T). These could be some of the reasons for the differences in mutation rates. The heterogeneity between study cohorts also underlines the need to validate new results in several larger MDS cohorts in order to widen our insight and understanding. There were no differences found between global methylation levels between mutated and wild-type patients, as already described by Ley *et al.* in AML.[5] Thus functional studies are needed to understand the role of mutations in *DNMT3A* in the pathogenesis of myeloid malignancies. In summary, mutations in *DNMT3A* occur in patients with MDS at a low frequency and further studies with larger cohorts are needed to evaluate a possible negative prognostic impact.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Jiang Y, Dunbar A, Gondek LP, Mohan S, Rataul M, O'Keefe C, et al. Aberrant DNA methylation is a dominant mechanism in MDS progression to AML. Blood. 2009; 113(6):1315-25.
2. Shen L, Kantarjian H, Guo Y, Lin E, Shan J, Huang X, et al. DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes. J Clin Oncol. 2010;28(4):605-13.
3. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. Lancet Oncol. 2009;10(3):223-32.
4. Broske AM, Vockentanz L, Kharazi S, Huska MR, Mancini E, Scheller M, et al. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. Nat Genet. 2009;41(11): 1207-15.
5. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, et al. DNMT3A mutations in acute myeloid leukemia. N Engl J Med. 2010;363(25):2424-33.
6. Walter MJ, Ding L, Shen D, Shao J, Grillot M, McLellan M, et al. Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. Leukemia. 2011;25(7):1153-8.
7. Thol F, Weissinger EM, Krauter J, Wagner K, Damm F, Wichmann M, et al. IDH1 mutations in patients with myelodysplastic syndromes are associated with an unfavorable prognosis. Haematologica. 2010;95 (10):1668-74.
8. Thol F, Friesen I, Damm F, Yun H, Weissinger EM, Krauter J, et al. Prognostic significance of ASXL1 mutations in patients with myelodysplastic syndromes. J Clin Oncol. 2011;29(18):2499-506.
9. Stadler M, Germing U, Kliche KO, Josten KM, Kuse R, Hofmann WK, et al. A prospective, randomised, phase II study of horse antithymocyte globulin vs rabbit antithymocyte globulin as immune-modulating therapy in patients with low-risk myelodysplastic syndromes. Leukemia. 2004;18(3):460-5.
10. Passweg JR, Giagounidis AA, Simcock M, Aul C, Dobbelstein C, Stadler M, et al. Immunosuppressive therapy for patients with myelodysplastic syndrome: a prospective randomized multicenter phase III trial comparing antithymocyte globulin plus cyclosporine with best supportive care--SAKK 33/99. J Clin Oncol. 2011;29 (3):303-9.
11. Hofmann WK, Ganser A, Seipelt G, Ottmann OG, Zander C, Geissler G, et al. Treatment of patients with low-risk myelodysplastic syndromes using a combination of all-trans retinoic acid, interferon alpha, and granulocyte colony-stimulating factor. Ann Hematol. 1999;78(3):125-30.
12. Porter J. Oral iron chelators: prospects for future development. Eur J Haematol. 1989; 43(4):271-85.
13. Thol F, Damm F, Lüdeking A, Winschel C, Wagner K, Morgan M, et al. Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. J Clin Oncol. 2011;29(21):2889-96.
14. Yan XJ, Xu J, Gu ZH, Pan CM, Lu G, Shen Y, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet. 2011;43(4):309-15.
15. Thol F, Damm F, Wagner K, Gohring G, Schlegelberger B, Hoelzer D, et al. Prognostic impact of IDH2 mutations in cytogenetically normal acute myeloid leukemia. Blood. 2010;116(4):614-6.
16. Wagner K, Damm F, Gohring G, Gorlich K, Heuser M, Schafer I, et al. Impact of IDH1 R132 mutations and an IDH1 single nucleotide polymorphism in cytogenetically normal acute myeloid leukemia: SNP rs11554137 is an adverse prognostic factor. J Clin Oncol. 2010;28(14):2356-64.