Frequency, onset and clinical impact of somatic *DNMT3A* mutations in therapy-related and secondary acute myeloid leukemia

Isabella Fried,[1,2] Claudia Bodner,[1] Monika M. Pichler,[1] Karin Lind,[1] Christine Beham-Schmid,[3] Franz Quehenberger,[4] Wolfgang R. Sperr,[5] Werner Linkesch,[1] Heinz Sill,[1] and Albert Wölfler[1]

[1]Division of Hematology, [2]Department of Dermatology, [3]Institute of Pathology, and [4]Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz, Austria; and [5]Division of Hematology and Hemostaseology, Department of Medicine I, Medical University of Vienna, Austria

ABSTRACT

The recent identification of *DNMT3A* mutations in *de novo* acute myeloid leukemia prompted us to determine their frequency, patterns and clinical impact in a cohort of 98 patients with either therapy-related or secondary acute myeloid leukemia developing from an antecedent hematologic disorder. We identified 24 somatic mutations in 23 patients with a significantly higher frequency in secondary acute myeloid leukemia (35.1%) as compared to therapy-related acute myeloid leukemia (16.4%, *P*=0.0486). *DNMT3A* mutations were associated with a normal karyotype and *IDH1/2* mutations, but did not affect survival. In contrast to *de novo* acute myeloid leukemia, most mutations did not affect arginine on position 882, but were predominantly found in the methyltransferase domain. All *DNMT3A* mutations identified in secondary acute myeloid leukemia were already present in the antecedent disorders indicating an early event. Reduction to homozygosity by uniparental disomy was observed in 2 patients with secondary acute myeloid leukemia during disease progression.

Key words: acute myeloid leukemia, secondary and therapy-related AML, *DNMT3A*, mutation.

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Introduction

Epigenetic deregulation of gene expression is increasingly recognized as being fundamental for the pathogenesis of acute myeloid leukemia (AML). Although the mechanisms by which these changes contribute to leukemogenesis are still not completely understood, the identification of mutations in several genes involved in epigenetic regulation has given new insight into these mechanisms. Recently, whole genome and exome sequencing led to the identification of recurrent somatic mutations in *DNMT3A* (encoding DNA methyltransferase 3A) in about 20% of patients with *de novo* AML.[1-3] DNMT3A is crucial for the methylation of unmodified DNA in CpG islands by converting cytosine to 5-methylcytosine which is associated with gene silencing.[4,5] *In vitro*, enzymatic assays showed a reduction in DNA methylation by mutated DNMT3A and overexpression of the two most frequent *DNMT3A* mutants (R882H and R882C) promoted proliferation in cell culture experiments.[3] Of clinical importance, *DNMT3A* mutations were associated with decreased overall survival.[1,3,6] These results strongly suggest a prominent pathogenetic role of *DNMT3A* mutations in AML.

Although most patients are diagnosed with *de novo* AML, in about 20-30% of cases AML presents as a sequelae of a previous disease. This group includes patients in whom AML develops after chemo- and/or radiotherapy for a primary, most often malignant disease. Furthermore, patients suffering from myelodysplastic syndromes or myeloproliferative neoplasms (MDS/MPN) have a substantial risk of transformation to AML. Both therapy-related (t-AML) as well as secondary AML (sAML) following an antecedent MDS/MPN are associated with low survival rates following conventional therapy including hematopoietic stem cell transplantation.[7-9] In some cases, t-AML and sAML share common cytogenetic and molecular alterations with poor risk *de novo* AML, such as loss of chromosome 5 and 7.[10,11] However, in a substantial proportion of patients, genetic alterations responsible for the dismal clinical course have not been established.

To determine the frequencies, patterns and clinical impact of *DNMT3A* mutations in patients with t-AML and sAML, we carried out mutational analysis of all coding exons of *DNMT3A* in a cohort of 98 patients using direct DNA sequencing. Furthermore, specimens from the antecedent hematologic disorder in sAML patients with mutated *DNMT3A* provided fresh insight into the onset of *DNMT3A* mutations and their role in leukemic transformation.

Design and Methods

Patients, DNA isolation and sequencing

Thirty-seven patients with sAML and 61 patients with t-AML were

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Correspondence: Albert Wölfler, Division of Hematology Medical University of Graz, Auenbruggerplatz 38 8036 Graz, Austria.
Phone: international +43.316.38584084. Fax: international +43.316.38514087. E-mail: albert.woelfler@medunigraz.at

included in this study. Diagnosis was made according to the French-American-British (FAB) classification. The majority of sAML patients had a history of antecedent MDS (n=27; 10 refractory anemia with excess blasts (RAEB); one RAEB in transformation; 16 refractory anemia) whereas the remaining sAML patients had a history of MPN (n=10; 5 primary myelofibrosis; one essential thrombocythemia; one polycythemia vera; 3 chronic myelomonocytic leukemia).

DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions from diagnostic peripheral blood or bone marrow samples after Ficoll enrichment yielding more than 90% blast cells. In patients with MDS or MPN, DNA was isolated from bone marrow biopsy specimens without further enrichment. For analysis of constitutional material, DNA was either isolated from buccal swabs or biopsies of non-hematologic tissues. cDNA was synthesized using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and the RevertAid™ H Minus First strand cDNA Synthesis Kit (Fermentas Life Science Europe, Bremen, Germany). This study was approved by the institutional review board of the Medical University of Graz, Austria, and was conducted in accordance with the Declaration of Helsinki.

All coding exons including the splice sites of *DNMT3A* were amplified by PCR (primers and detailed PCR conditions are given in the *Online Supplementary Table S1*). Sequencing of both strands was performed with the ABI PRISM 3730 (Applied Biosystems, Foster City, CA, USA) and mutations were identified using SeqScape®Software v2.5 (Applied Biosystems, Foster City, CA, USA). Each identified mutation was confirmed at least once by an independent sequence analysis using genomic DNA. Analysis of mutations in IDH1/2 was performed as previously described.[12]

SNP array analysis in patients with homozygous DNMT3A mutations

Whole genome single nucleotide polymorphism (SNP) analysis was performed to determine copy number variations and regions with uniparental disomy in patients with homozygous *DNMT3A* mutations. For this purpose, Affymetrix human genome-wide SNP 6.0 arrays (Affymetrix, Santa Clara, CA, USA) were used according to the manufacturer's protocol and results were analyzed in comparison to 60 HapMap individuals using Genotyping Console™ Version 4.0.

Statistical analysis

Differences in characteristics of patients with or without *DNMT3A* mutations were calculated using a two-sided Fisher´s exact or Mann-Whitney test. The Kaplan-Meier method was applied to generate the survival curves and differences were assessed by log rank analysis. R 2.13.0 software (*www.r-project.org*) was used for the analyses.

Results and Discussion

Frequency and patterns of DNMT3A mutations in t- and sAML

Of 98 t-AML and sAML samples in this study, a

Table 1. Clinical characteristics of t-AML and sAML patients with *DNMT3A* mutations.

UPN	Leukemia	Age at AML diagnosis (years)	Primary disease	Karyotype	DNMT3A mutation
1522	t-AML	65	Non-Hodgkin's lymphoma	46,XY	R882H
5038	t-AML	75	Prostate and colon cancer	NA	V636M
5830	t-AML	75	AML	46,XX	R882H
5413	t-AML	80	Breast cancer	NA	R882C
6874	t-AML	66	Lymphoma and colon cancer	47,XX,+4	c.1123-2
6505	t-AML	80	Multiple myeloma	46,XX	Y735C
4890	t-AML	73	Non-Hodgkin's lymphoma	45,XY, inv(3)(q21q26), -7	W581C
4729	t-AML	72	Hepatocellular carcinoma	46,XY	L754R
5630	t-AML	51	Non-Hodgkin's lymphoma	46,XX,der(7)(7pter→7q22::3q26-3qter)	S352D
4734	t-AML	61	Uterine cancer	46,XX,t(11;19)(q23;p13.1)	P849R
2331	sAML	68	RAEB-T	46,XX	F752L
3205	sAML	79	RAEB	46,XY	R882C
5488	sAML	62	RAEB	46,XY	R882C
5790	sAML	65	RA	NA	R882H
5489	sAML	64	CMML	46,XY	R882H
6285	sAML	78	MF	46,XX	R882H
4544	sAML	54	RAEB	46,XY	R882H
5671	sAML	68	MF	NA	L889P, F731C
5353	sAML	53	RA	46,XX,der(7),t(3;7)(q26;p2?)	W753R
2028	sAML	75	RA	NA	W795C
4881	sAML	70	RAEB	46,XX	I655A
3967	sAML	65	RA	45,XY,-7	G543A
5390	sAML	59	MF	43-47,XX,+der(9),del(9)(q22q23)	A910V

UPN: universal patient number; NA: not applicable; RAEB: refractory anemia with excess blasts; RAEB-T: refractory anemia with excess blasts in transformation; RA: refractory anemia; MF: myelofibrosis; CMML: chronic myelomonocytic leukemia.

DNMT3A mutation was identified in a total of 23 (23.6%). One patient displayed two mutations (clinical details of patients with mutations are given in Table 1; a comparison of clinical characteristics in *DNMT3A*-mutated *vs.* non-mutated patients is given in the *Online Supplementary Table S2*). The frequency of *DNMT3A* mutations in this cohort is, therefore, similar to patients with *de novo* AML, which was reported to be 22.1%,[1] 20.5%[3] and 17.8%.[6] However, when assessing sAML and t-AML patients of our cohort separately, the percentage of patients with a *DNMT3A* mutation was higher in sAML (13 of 37, 35.1%) than in t-AML (10 of 61, 16.4%; *P*=0.0486). By analyzing patients for whom information on cytogenetics was available, the frequency of mutations was significantly higher in patients with a normal karyotype (11 of 25 patients, 44%) as compared to patients with cytogenetic aberrations (7 of 50, 14%; *P*=0.00841). Furthermore, as has been reported for *de novo* AML,[1] mutations in the *IDH1/2* gene were associated with mutated *DNMT3A* in our cohort of t-AML and sAML patients (5 of 23, 21.7% *vs.* 2 of 75, 2.7%, *P*=0.00731; *Online Supplementary Table S2*).

The mutations identified in this study were made up of 23 missense and one splice site mutation located upstream of exon 10 (c.1123-2 A>G; Table 1 and *Online Supplementary Figure S1*). Although the hot spot mutation affecting arginine on position 882 was also the most frequent mutation in our study (3 in t-AML and 6 in sAML), the majority of mutations in *DNMT3A* (n=15) affected other amino acid residues (Table 1 and *Online Supplementary Figure S1*). This is in contrast to the findings in *de novo* AML, where according to all published studies[1,3,6] 116 out of 176 mutations were affecting R882 (*P*=0.01203 as compared to our cohort). These results suggest a somewhat different pattern of *DNMT3A* mutations in t-AML and sAML as compared to *de novo* AML. Since most of our identified mutations (S352N, W581C, V636M, I655A, F731C, Y735C, F752L, W753G, L754R, W795C, P849R, L889P, A910V) have not been reported previously, we tested constitutional DNA to prove the somatic nature of these mutations (Figure 1 and *Online Supplementary Figure S1*). In all but one case, in which we had no access to constitutional material, we were able to delineate the somatic origin of the mutations. In the remaining case, the affected amino acid is highly conserved among species and is not reported to constitute single nucleotide polymorphisms in public databases. Mutation of the acceptor

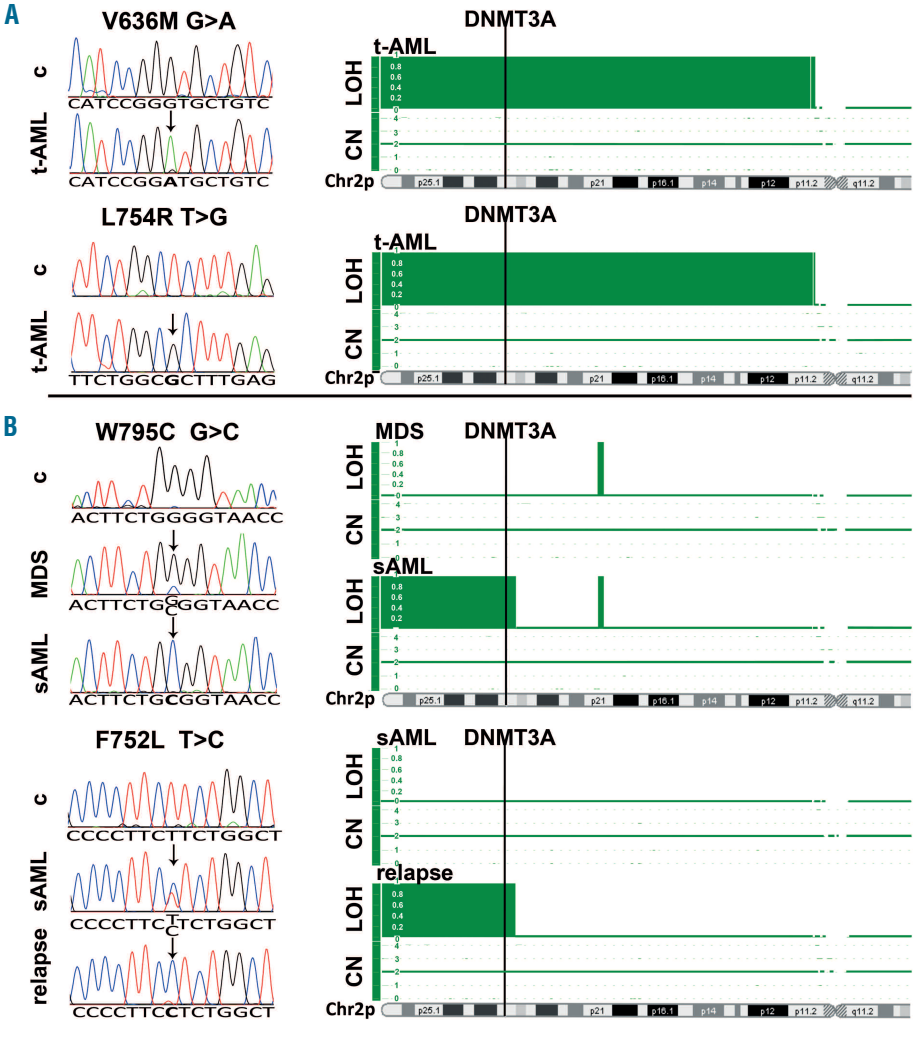

Figure 1. Uniparental disomy of 2p23.3 results in homozygous *DNMT3A* mutations in distinct t-AML and sAML patients. (A) Electropherograms of two homozygous *DNMT3A* mutations in t-AML samples showing no mutation in the corresponding constitutional material. SNP-array analysis revealed a copy-neutral loss of heterozygosity of 2p11-2pter that harbors the *DNMT3A* gene locus in both t-AML samples. (B) Electropherograms of one patient (upper panel) with a somatic heterozygous mutation in MDS who developed a homozygous W795C mutation during the progression to sAML. A second patient (lower panel) showed a somatic heterozygous mutation at sAML diagnosis, but displayed a homozygous F752L mutation at the time of sAML relapse after autologous stem cell transplantation. Corresponding SNP-array analysis demonstrated a copy-neutral loss of heterozygosity of 2p23.3-2pter in both samples with the homozygous mutations, but not in the samples with heterozygous *DNMT3A* mutations. c: constitutional material; LOH: loss of heterozygosity; CN: copy number; Chr2p: short arm of chromosome 2.

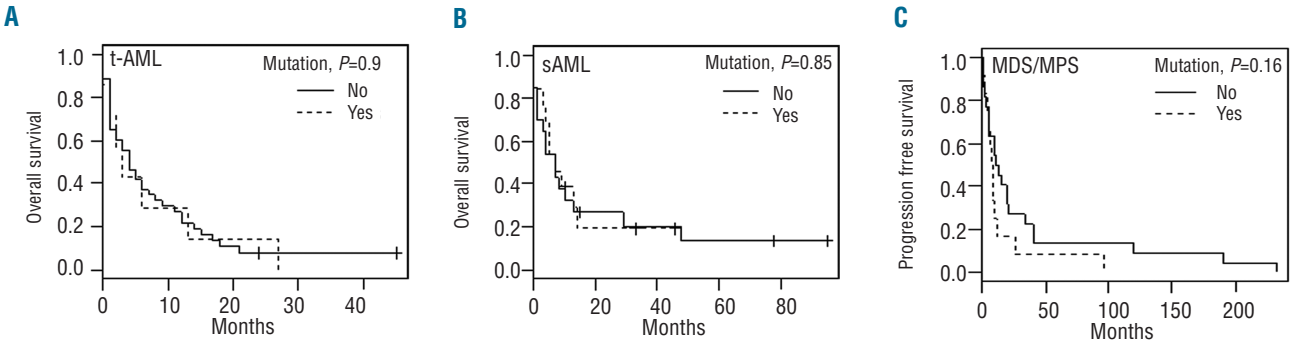

Figure 2. Survival analysis of 82 t-AML and sAML patients with respect to their *DNMT3A* mutational status. (A) The overall survival of t-AML patients with *DNMT3A* mutations (n=7) as compared to non-mutated patients (n=42). (B) The overall survival of sAML patients with *DNMT3A* mutations (n=13) as compared to patients without *DNMT3A* mutations (n=20). (C) Kaplan-Meier plot of time to progression to sAML with respect to the *DNMT3A* mutation status in patients diagnosed with MDS/MPN.

splice site resulted in the use of an alternative 3' splice site upstream, thereby inserting a 40-bp intronic sequence in between exon 9 and exon 10 in the patient's mRNA (*Online Supplementary Figure S1A*, lower left panel). As a consequence, a premature stop codon is introduced that is predicted to lead to a truncated protein with 432 amino acids lacking the zinc finger and methyltransferase domains.

DNMT3A *mutations occur early in MDS/MPN and reduction to homozygosity is occasionally associated with disease progression*

Two recent studies reported an incidence of 8 and 10% of *DNMT3A* mutations in MDS and MPN, respectively,[13,14] substantially lower than the percentage of mutated samples in our sAML cohort (35.1%). One explanation for this discrepancy might be the acquisition of *DNMT3A* mutations during MDS/MPN progression resulting in transformation to sAML. To test this hypothesis, we analyzed DNA from bone marrow biopsies at initial diagnosis of MDS/MPN in 10 patients with mutated sAML samples. In all patients, the corresponding mutation could already be detected at that time indicating that *DNMT3A* mutations are an early event in the pathogenesis of these malignancies. Interestingly, we identified one MDS patient with a heterozygous mutation that became homozygous during progression to sAML (Figure 1B, upper panel). Another patient, who was still heterozygous for the mutation at sAML diagnosis displayed homozygosity for the mutation at the time of sAML relapse after autologous stem cell transplantation (Figure 1B, lower panel). Remarkably, this patient also showed a homozygous *IDH2* mutation at relapse of the sAML, which was recently reported by our group.[12] These results indicate that in some MDS/MPN patients, progression of disease is associated with the loss of the *DNMT3A* wild-type allele.

Two patients with t-AML also showed a homozygous *DNMT3A* mutation (Figure 1A) which, together with the 2 sAML patients, is a substantial number considering that homozygous *DNMT3A* mutations have rarely been documented in AML.[1,3] To identify the mechanisms leading to homozygosity of mutant *DNMT3A*, we performed copy-number variation analysis and heterozygosity mapping in all 4 patients using Affymetrix human genome-wide SNP 6.0 arrays. We detected a copy-neutral loss of heterozygosity of 2p23.3-2pter in both sAML patients and a copy-neutral loss of heterozygosity of 2p11-2pter in both t-AML patients (Figure 1C). Since the *DNMT3A* gene is located on 2p23.3, these results implicate uniparental disomy (UPD) of the region carrying the mutated *DNMT3A* allele in all these patients. Except for the patient who also showed a homozygous *IDH2* mutation and UPD of the involved region on 15q as well as a deletion of 7q,[14] UPD of 2p was the only detected aberration in the remaining 3 patients using SNP array analysis.

Clinical impact of DNMT3A *mutations in t-AML and sAML*

In *de novo* AML, *DNMT3A* mutations are associated with an adverse prognosis.[1,3,6] However, while uni- and multivariate analysis identified well-known risk factors for poor survival in our cohort, such as cytogenetic risk and white blood cell counts at AML diagnosis, the presence of *DNMT3A* mutations did not affect overall survival in t-AML and sAML patients (Figure 2A and B). Since t-AML and sAML are known to be associated with poor survival as compared to *de novo* AML and many of these patients display poor risk cytogenetics, it may not be surprising that *DNMT3A* mutations do not further add to the poor prognosis in this cohort of patients. Mutated patients showed a shorter interval between MDS/MPN diagnosis and transformation to sAML (median time to progression 7.5 *vs.* 12 months), which is in accordance with the findings of Walter *et al.* who reported a more rapid progression to AML in MDS patients with *DNMT3A* mutations.[13] However, in our cohort the difference was not statistically significant (P=0.17).

In summary, this study demonstrated *DMNT3A* mutations in about one sixth of patients with t-AML. In sAML, even one out of 3 patients was carrying a mutation in *DNMT3A*. The respective mutations occurred early in the myelodysplastic or myeloproliferative phase of the disease and reduction to homozygosity by uniparental disomy of the genetic region involved was found in some patients during disease progression. *DNMT3A* mutations did not constitute an additional adverse risk factor in these poor prognostic subtypes of AML.

Authorship and Disclosures

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