based chromosomal analysis have important clinical impact in acute myeloid leukemia. J Clin Oncol. 2009;27(31):5219-26.
7. Walter MJ, Payton JE, Ries RE, Shannon WD, Deshmukh H, Zhao Y, et al. Acquired copy number alterations in adult acute myeloid leukemia genomes. Proc Natl Acad Sci USA. 2009;106(31):12950-5.
8. Bajaj R, Xu F, Xiang B, Wilcox K, Diadamo AJ, Kumar R, et al. Evidence-based genomic diagnosis characterized chromosomal and cryptic imbalances in 30 elderly patients with myelodysplastic syndrome and acute myeloid leukemia. Mol Cytogenet. 2011;4:3.
9. Streubel B, Sauerland C, Heil G, Freund M, Bartels H, Lengfelder E, et al. Correlation of cytogenetic, molecular cytogenetic, and clinical findings in 59 patients with ANLL or MDS and abnormalities of the short arm of chromosome 12. Br J Haematol. 1998;100(3):521-33.
10. Heinrichs S, Kulkarni RV, Bueso-Ramos CE, Levine RL, Loh ML, Li C, et al. Accurate detection of uniparental disomy and microdeletions by SNP array analysis in myelodysplastic syndromes with normal cytogenetics. Leukemia. 2009;23(9):1605-13.
11. Kolquist KA, Schultz RA, Furrow A, Brown TC, Han JY, Campbell LJ, et al. Microarray-based comparative genomic hybridization of cancer targets reveals novel, recurrent genetic aberrations in the myelodysplastic syndromes. Cancer Genet. 2011;204(11):603-28.
12. Rucker FG, Bullinger L, Schwaenen C, Lipka DB, Wessendorf S, Frohling S, et al. Disclosure of candidate genes in acute myeloid leukemia with complex karyotypes using microarray-based molecular characterization. J Clin Oncol. 2006;24(24):3887-94.

Mutations in *DNMT3A* and loss of RKIP are independent events in acute monocytic leukemia

Methylation of DNA in CpG-rich islands is a key event in the regulation of tissue- and context-specific gene expression. Thereby, DNA methyltransferase 3A (*DNMT3A*) plays a pivotal role by converting cytosine to 5-methylcytosine.[1] Recently, *DNMT3A* mutations have been described at high frequency in acute myeloid leukemia (AML), particularly with a monocytic phenotype.[2-4] In *in vitro* assays, mutated *DNMT3A* induced aberrant DNA methylation and promoted cellular proliferation. RAF kinase inhibitor protein (RKIP) negatively regulates the RAS-mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway.[5] Several solid neoplasms show decreased or absent expression of RKIP and its role as a metastasis suppressor has been firmly established in animal models and studies of human tumors.[5] We recently demonstrated that RKIP acts as a tumor suppressor in hematopoietic cells and that loss of RKIP expression occurs frequently in AML.[6,7] Similar to mutated *DNMT3A*, RKIP loss is linked to AMLs with a monocytic phenotype. We, therefore, asked whether loss of RKIP and mutations in *DNMT3A* correlate in this particular subtype.

For PCR and direct sequencing of *DNMT3A*, its coding sequences and splice sites comprising 22 exons were analyzed as previously described[5] in leukemic specimens of 36 patients diagnosed with acute monocytic leukemia; patients were classified in subgroups M4 and M5 according to the French-American-British (FAB) classification. Cytogenetic information was available in 32 patients with 4 of 32 (12.5%) karyotypes conferring good, 21 of 32 (65.5%) intermediate, and 7 of 32 (22%) adverse risk. Median age at diagnosis was 55.5 years (range 18-80 years), median white blood cell (WBC) count 55.5x10^9/L (range 5-445x10^9/L). This cohort has also been characterized for RKIP expression, showing its loss in 17 of 36 (47%) patients as determined at protein and mRNA level using Western blot and quantitative real-time polymerase chain reaction (PCR), respectively.[7] Screening for mutations in *NRAS* and *KRAS* (codons 12, 13 and 61) as well as in *NPM1* (exon 12) has been performed as previously described.[7,8] Detection of *FLT3* internal tandem duplications (ITD) and tyrosine kinase domain (TKD) mutations was performed using the *FLT3* Mutation Assay (*InVivo*Scribe Technologies, San Diego, CA, USA) according to the manufacturer's protocol. Informed consent was obtained from all individuals and the study was approved by the institutional review board of the Medical University of Graz, Austria. Statistical correlations were calculated by Fisher's exact (for correlation of *DNMT3A* with *RKIP*, *FLT3* and *NPM1*) and by Mann-Whitney-Wilcoxon test (for correlation of *DNMT3A* with cytogenetics, WBC count and age at diagnosis), using R 2.15.1 (*http://www.r-project.org*).

We detected mutations in *DNMT3A* in 16 of 36 (44%) patients with monocytic AML. This high frequency is consistent with previous reports on *DNMT3A* mutations in this AML subtype.[2,4] Fifteen of these mutations constituted the recently described hot spot mutations R882H and R882C, respectively.[2,4] In one sample, we detected an R676W substitution, a *DNMT3A* mutation that has not yet been described. Its somatic origin could be proven by analysis of constitutional material (Figure 1). This substitution was predicted to be "disease causing" by MutationTaster[9] and "damaging" by Sorting Intolerant From Tolerant (SIFT).[10] WBC counts at diagnosis were significantly higher in patients with mutant as compared to wild-type *DNMT3A* (77x10^9/L *vs*. 51x10^9/L; *P*=0.040), whereas no significant difference could be observed for cytogenetic risk groups and patients age at diagnosis (*data not shown*). In the cohort investigated, *DNMT3A* mutations were associated with alterations of *NPM1* as 11 of 14 (79%) *DNMT3A* mutated patients also harbored a *NPM1* mutation compared to 6 of 20 (30%) *DNMT3A*

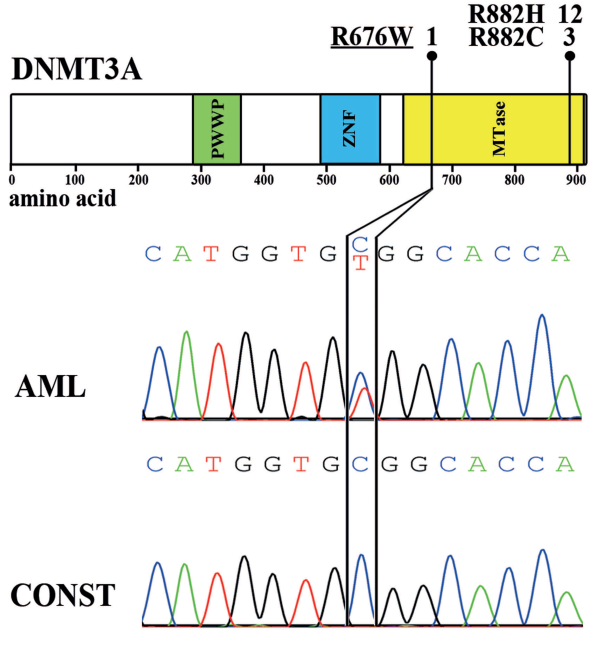

Figure 1. R676W is a novel somatic DNMT3A mutation. Number of distinct mutations detected in this study and their locations within the DNMT3A protein. The novel R676W substitution is caused by the heterozygous C2026T substitution as highlighted with black bars in the electropherogram of leukemic DNA. Absence of this mutation in constitutional material proves its somatic origin. PWWP: proline-tryptophan-tryptophan-proline domain; ZNF: zinc finger domain; MTase: methyltransferase domain; CONST: constitutional material.

Figure 2. Molecular correlations of *DNMT3A* mutations in monocytic AML. Heatmap showing the occurrence of *DNMT3A*, *NPM1*, *FLT3* and *RAS* mutations as well as RKIP loss in 36 patients with monocytic AML. Exon 12 alterations in *NPM1* were significantly enriched in patients with mutant *DNMT3A* (P=0.014), whereas RKIP loss and aberrations in *FLT3* did not correlate with *DNTM3A* mutations. Note that RAS status was excluded from statistical testing due to the low number of mutated cases.

wild-type patients (P=0.014). The rates of *FLT3*-ITD and mutations in the *FLT3*-TKD were found to be similar in *DNMT3A* mutated and *DNMT3A* wildtype cases. Notably, only 3 of 34 (9%) patients demonstrated mutations in *NRAS* or *KRAS*, which precluded statistical testing (Figure 2).

Importantly, mutations in *DNMT3A* did not correlate with loss of the RKIP protein (P=0.99) as calculated by Fisher's exact test (Figure 2). This finding is of interest as the causative mechanisms of RKIP silencing have not yet been clarified. We previously screened for mutations and copy number variations in the RKIP gene as well as for promoter methylation, but did not find any abnormalities.[7] Interestingly, DNMT3A has been shown to induce both hyper- and hypomethylation at distinct loci including non-promoter regions resulting in direct and indirect alteration of gene expression profiles.[11,12] However, the lack of correlation between these two aberrations observed in this study suggests that RKIP silencing is not mediated by mutations in *DNMT3A*.

Taken together, we confirmed a high frequency of *DNMT3A* mutations in monocytic AML and described one novel somatic substitution. Mutant *DNMT3A* occurred independently of the RKIP expression status suggesting other, hitherto unknown mechanisms responsible for loss of RKIP in AML.

Isabella Fried,[1,2] Albert Wölfler,[1] Franz Quehenberger,[3] Gerald Hoefler,[4] Heinz Sill,[1] and Armin Zebisch[1]

[1]Division of Hematology; [2]Department of Dermatology; [3]Institute of Medical Informatics, Statistics and Documentation; and [4]Institute of Pathology, Medical University of Graz, Graz, Austria

Correspondence: Armin Zebisch, Division of Hematology, Medical University of Graz, Auenbruggerplatz 38, 8036 Graz, Austria. Phone: international +43.316.38514086. Fax: international +43.316.38514087. E-mail: armin.zebisch@medunigraz.at

Key words: acute monocytic leukemia, DNMT3A mutations, RKIP loss.

Funding: this study was supported by Leukämiehilfe Steiermark. Armin Zebisch was further supported by the START-Funding-Program of the Medical University of Graz, Graz, Austria.

Citation: Fried I, Wölfler A, Quehenberger F, Hoefler G, Sill H, and Zebisch A. Mutations in DNMT3A and loss of RKIP are independent events in acute monocytic leukemia. Haematologica 2012;97(12):1936-1937.doi:10.3324/haematol.2012.068429

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. Attwood JT, Yung RL, Richardson BC. DNA methylation and the regulation of gene transcription. Cell Mol Life Sci. 2002;59(2):241-57.
2. 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.
3. Fried I, Bodner C, Pichler MM, Lind K, Beham-Schmid C, Quehenberger F, et al. Frequency, onset and clinical impact of somatic DNMT3A mutations in therapy-related and secondary acute myeloid leukemia. Haematologica. 2012;97(2):246-50.
4. 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.
5. Matallanas D, Birtwistle M, Romano D, Zebisch A, Rauch J, von Kriegsheim A, et al. Raf family kinases: old dogs have learned new tricks. Genes Cancer. 2011;2(3):232-60.
6. Zebisch A, Haller M, Hiden K, Goebel T, Hoefler G, Troppmair J, et al. Loss of RAF kinase inhibitor protein is a somatic event in the pathogenesis of therapy-related acute myeloid leukemias with C-RAF germline mutations. Leukemia. 2009;23(6):1049-53.
7. Zebisch A, Wolfler A, Fried I, Wolf O, Lind K, Bodner C, et al. Frequent loss of RAF kinase inhibitor protein expression in acute myeloid leukemia. Leukemia. 2012;26(8):1842-9.
8. Pasqualucci L, Liso A, Martelli MP, Bolli N, Pacini R, Tabarrini A, et al. Mutated nucleophosmin detects clonal multilineage involvement in acute myeloid leukemia: Impact on WHO classification. Blood. 2006;108(13):4146-55.
9. Schwarz JM, Rodelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. Nat Methods. 2010;7(8):575-6.
10. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009;4(7):1073-81.
11. Challen GA, Sun D, Jeong M, Luo M, Jelinek J, Berg JS, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet. 2011;44(1):23-31.
12. Wu H, Coskun V, Tao J, Xie W, Ge W, Yoshikawa K, et al. Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science. 2010;329(5990):444-8.