Genetic characterization of acquired aplastic anemia by targeted sequencing

Aplastic anemia (AA) is a rare but life-threatening bone marrow failure syndrome; diagnosis is based on cytopenias in peripheral blood and hypocellularity in bone marrow. The distinction between AA and hypocellular myelodysplastic syndrome (MDS) is often difficult to verify, and AA evolves into MDS or AML at a 10-year cumulative incidence of 3.1-9.6%.[1,2] As AA patients often respond to immunosuppressive therapy, an immune pathophysiology is widely assumed.[3] The evolution of clonal cytogenetic aberrations in hematopoietic cells and the association with clonal paroxysmal nocturnal hemoglobinuria (PNH) suggest that some patients have a clonal hematopoietic disease.[4] Data by Walter and colleagues show that 74% of MDS patients harbor a mutation in at least one of 94 candidate genes, while mutations can still be found in 68% of MDS patients when a core set of 25 genes is analyzed.[5] We hypothesized that mutations that are found in MDS patients may also be present in patients with AA and, therefore, evaluated the mutation profile of 42 genes in AA patients.

Thirty-eight patients with aplastic anemia who were diagnosed and treated in our institution were included in our study. Patients' characteristics are described in detail in the *Online Supplementary Appendix and Online Supplementary Table S1*. Written informed consent was obtained according to the Declaration of Helsinki, and the study was approved by the institutional review board. Coding exons of the 33 most frequently mutated genes in myeloid malignancies (*Online Supplementary Table S2*) were sequenced on the SOLiD™ system (Life Technologies, Darmstadt, Germany) as described in the *Online Supplementary Appendix*. Nine other genes mutated in myeloid malignancies including TERT, all candidate mutations from next generation sequencing, exon 13 of *ASXL1*, and exons 3 to 11 of *TET2*, were sequenced by Sanger sequencing or analyzed by fragment analysis (*Online Supplementary Table S2*). Confirmed mutations were also evaluated in germline DNA ($CD3^+$ T cells from peripheral blood or hair follicles).

Eleven of 38 patients had moderate, 12 severe, and 15 very severe AA. Median age of AA patients at diagnosis was 30 years (range 9-79 years). Three patients (8%) had abnormal cytogenetics (*Online Supplementary Table S1*). In

Table 1. Mutations in aplastic anemia patients. Sequence numbering is according to the protein sequence of Ensembl protein ID ENSP00000266058 (SLIT1), ENSP00000282030 (SETBP1) and ENSP00000364839 (ASXL1), ENSP00000217026 (MYBL2), ENSP00000442788 (TET2), of genome build GRCh37, release 73.

Characteristic	Somatic mutations		Germline SNVs	
Patient ID	1	2	3	4
Age at diagnosis (years)	61	14	42	45
Age at sampling (years)	61	28	58	45
Sex	M	M	F	F
Severity	VSAA	SAA	MAA	SAA
Treatment	ATG/	ATG/CSA, alloHSCT	CSA, ATG/CSA	Syngeneic HSCT
Time from diagnosis to first treatment (months)	0.5	16	1	4
Best response (CR, PR)	CR	CR	PR	CR
Time from last treatment to last follow up (years)	1.9	8.3	4.3	9
PNH clone (granulocytes, %)	4	1.4	0	0
Telomere length (average T/S ratio)	unknown	unknown	1.25	unknown
Source of DNA	BM	PB	BM	PB
Karyotype	46,XY	46,XY	46,XX, developed trisomy 8	46,XX
Affected gene	SLIT1	SETBP1, ASXL1	MYBL2	TET2
Variation, nucleotide level	c.1252C>A	c.2602G>A c.1934dupG	c.844T>C	c.2981A>G
Variation, protein level	p.Q418K	p.D868N**, p.G645fs***	p.S282P	p.H994R*
Germline affected	No	No	Yes	Yes
Variant allele frequency (%)	50§	35§ 25§	38.7	50
Survival from diagnosis (years), status	1.5, alive	21.6, alive	16.8, alive	7.5, alive

*M: male; F: female; SAA: severe aplastic anemia; VSAA: very severe aplastic anemia; MAA: moderate aplastic anemia; ATG: anti-thymocyte globulin; CSA: ciclosporin; alloHSCT: allogeneic hematopoietic stem cell transplantation; PB: peripheral blood; BM: bone marrow; n.a.: not applicable. *previously identified as somatic SNV COSM1426210 **previously identified as somatic SNV in SETBP1, COSM1318401 ***previously identified as somatic insertion in ASXL1, COSM34210 §estimated from Sanger sequencing trace.*

17 patients (44.7%), a GPI-deficient clone suggesting PNH/AA overlap syndrome was present. Twenty-two patients underwent allogeneic or syngeneic transplantation. The median duration of follow up from diagnosis of patients alive was seven years. Thirty-five of 38 patients were alive at last follow up. In support of the diagnosis of AA, telomeres in peripheral blood leukocytes were significantly shorter in AA patients (n=13) than in age-matched healthy controls (n=20) (median age AA, 44 years; median age healthy controls, 43 years, *P*=0.43; mean T/S ratio AA patients 1.43, mean T/S ratio healthy controls, 2.48, *P*<0.001) (*Online Supplementary Figure S1*). Next generation sequencing yielded an average coverage of 2015 reads per amplicon. In total, 3 somatic mutations were identified in 2 patients in the examined MDS candidate genes (5.3%) (Table 1 and *Online Supplementary Figure S2*). One patient had a somatic missense mutation in SLIT1, and one patient with severe AA had two somatic mutations, one missense mutation in *SETPB1* (D868N) and one frameshift mutation in *ASXL1* (*G645fs*). This patient was diagnosed with severe AA at the age of 14 years and received four courses of immunosuppressive therapy. After the first cycle of ATG, the patient was in remission for four years. There was no durable response upon subsequent cycles of immunosuppressive therapy. Eleven years after diagnosis, treatment with SCF and G-CSF was started, which induced a partial remission. Two years later the patient developed progressive thrombocytopenia and had signs of multilineage dysplasia in bone marrow. The patient received an allogeneic transplantation, and is alive 8.3 years later with normal blood counts. The current analysis was performed on cells harvested shortly before transplantation. This patient most likely had progressed to MDS at time of investigation. The patient with an *SLIT1* mutation had very severe AA and did not respond to the first course of rabbit ATG (3.75 mg/kg Days 1-5) plus CSA. Fourteen months later a second course of horse ATG (40 mg/kg Days 1-4) plus CSA was started; the patient responded well and is in ongoing remission.

Two additional patients were identified with germline single nucleotide variants (SNV) in *MYBL2* and *TET2*, respectively (Table 1). One patient had a previously unknown missense SNV in *MYBL2*, predicted to be possibly damaging to the protein with a score of 0.93 by polyphen-2,6 who developed trisomy 8 in the course of the disease; the other patient had a missense SNV in *TET2*, which has previously been described as somatic SNV in a different patient (COSM1426210). The patient with *MYBL2* SNV did not respond to the first course of rabbit ATG (3.5 mg/kg Days 1-5) plus CSA, but responded to the second course of rabbit ATG (3.5 mg/kg Days 1-5) plus CSA initiated 19 months later and is in partial remission (low platelets). The other patient with germline *TET2* SNV received a syngeneic transplant after conditioning with cyclophosphamide (50 mg/kg Days -5 to -2) without preceding immunosuppressive therapy and is in remission nine years after transplantation. In summary, somatic mutations of candidate genes of myeloid malignancies are rarely found in AA at the chosen cut off of 15% allele frequency. Whether germline mutations in *TET2* or *MYBL2* may predispose to AA needs to be addressed in future studies.

ASXL1 and *TET2* are the most frequently mutated genes in MDS.[7,8] *ASXL1* mutations have been identified previously at low allelic ratios of 8% and 9% in 2 AA patients, respectively.[9] We excluded mutations from further validation with allelic burden below 15% as these mutations cannot be validated by Sanger sequencing and their pathogenic role is unclear. *SETBP1* mutations are recurrent in MDS at a low frequency,[10] and are associated with atypical chronic myeloid leukemia (CML) and *ASXL1* mutations as in our patient.[11] *MYBL2* is a candidate tumor suppressor gene that is located in the commonly deleted region of MDS patients with del20q,[12] and haploinsufficiency of this gene predisposes to MDS in a mouse model.[13] SLIT1 mutations were first reported in AA patients by Saito *et al*.[14] The SLIT/ROBO pathway regulates axon guidance and has been implicated as a tumor suppressor.[15]

Considering the 25 genes that were recently analyzed by Walter *et al*.[5] in MDS patients, and which were also included in our analysis, somatic mutations were found in 68% of MDS patients but only 2 (5.3%) AA patients, one of whom had likely progressed to MDS. Patients' characteristics of the Walter study and our study are listed in *Online Supplementary Table S3*. We, therefore, suggest that mutation analysis of myelodysplasia-related genes may help to distinguish AA from MDS in ambiguous cases and may identify patients who are at risk for MDS-progression. Whether sequential mutation analysis provides prognostic information and is superior to cytogenetic analysis will be analyzed prospectively in the future. Recently, germline mutations in thrombopoietin have been identified in familial aplastic anemia.[16] In addition, 2 patients with AA have been described who had mutations in *MPL*, the receptor of thrombopoietin.[17] However, we have not identified *MPL* mutations in our patient cohort.

In summary, we identified somatic mutations in 5.3% of AA patients, of whom one had progressed to MDS. Our analysis suggests that mutations in myeloid malignancy-related genes are rare in AA patients.

Michael Heuser,[1] Carola Schlarmann,[1] Vera Dobbernack,[1] Victoria Panagiota,[1] Lutz Wiehlmann,[2] Carolin Walter,[3] Fabian Beier,[4] Patrick Ziegler,[4] Haiyang Yun,[1] Sofia Kade,[1] Aylin Kirchner,[1] Liu Huang,[1,5] Christian Koenecke,[1] Matthias Eder,[1] Tim H. Brümmendorf,[4] Martin Dugas,[3] Arnold Ganser,[1] and Felicitas Thol[1]

[1]Department of Hematology, Hemostasis, Oncology, and Stem Cell Transplantation, Hannover Medical School, Germany; [2]Clinical Research Group, Department of Pediatric Pneumology, Allergology and Neonatology, Hannover Medical School, Germany; [3]Institute of Medical Informatics, Münster University, Germany; [4]Department of Oncology, Hematology and Stem Cell Transplantation, RWTH Medical School, Aachen, Germany; and [5]Department of Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China

Funding: we thank Anke Franzke for indicating potential study participants. This study was supported by grants 109003, 110284, 110292 and 111267 from Deutsche Krebshilfe; grant DJCLS R10/22 and R13/14 from the Deutsche-José-Carreras Leukämie-Stiftung e.V; the German Federal Ministry of Education and Research grant 01EO0802 (IFB-Tx); DFG grants HE 5240/4-1, HE 5240/5-1, and TH 1779/1-1 and by the Dieter Schlag-Stiftung.

The online version of this article has a Supplementary Appendix.

Correspondence: heuser.michael@mh-hannover.de

doi:10.3324/haematol.2013.101642

Key words: MDS-related mutations, aplastic anemia.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Socie G, Henry-Amar M, Bacigalupo A, Hows J, Tichelli A, Ljungman P, et al. Malignant tumors occurring after treatment of

aplastic anemia. European Bone Marrow Transplantation-Severe Aplastic Anaemia Working Party. N Engl J Med. 1993;329(16): 1152-7.
2. Tichelli A, Schrezenmeier H, Socie G, Marsh J, Bacigalupo A, Duhrsen U, et al. A randomized controlled study in patients with newly diagnosed severe aplastic anemia receiving antithymocyte globulin (ATG), cyclosporine, with or without G-CSF: a study of the SAA Working Party of the European Group for Blood and Marrow Transplantation. Blood. 2011;117(17):4434-41.
3. Scheinberg P, Young NS. How I treat acquired aplastic anemia. Blood. 2012;120(6):1185-96.
4. Afable MG 2nd, Wlodarski M, Makishima H, Shaik M, Sekeres MA, Tiu RV, et al. SNP array-based karyotyping: differences and similarities between aplastic anemia and hypocellular myelodysplastic syndromes. Blood. 2013;117(25):6876-84.
5. Walter MJ, Shen D, Shao J, Ding L, White BS, Kandoth C, et al. Clonal diversity of recurrently mutated genes in myelodysplastic syndromes. Leukemia. 2013;27(6):1275-82.
6. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods. 2010;7(4):248-9.
7. 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.
8. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet. 2009;41(7):838-42.
9. Lane AA, Odejide O, Kopp N, Kim S, Yoda A, Erlich R, et al. Low frequency clonal mutations recoverable by deep sequencing in patients with aplastic anemia. Leukemia. 2013;27(4):968-71.
10. Thol F, Suchanek KJ, Koenecke C, Stadler M, Platzbecker U, Thiede C, et al. SETBP1 mutation analysis in 944 patients with MDS and AML. Leukemia. 2013;27(10):2072-5.
11. Meggendorfer M, Bacher U, Alpermann T, Haferlach C, Kern W, Gambacorti-Passerini C, et al. SETBP1 mutations occur in 9% of MDS/MPN and in 4% of MPN cases and are strongly associated with atypical CML, monosomy 7, isochromosome i(17)(q10), ASXL1 and CBL mutations. Leukemia. 2013;27(9):852-60.
12. Heinrichs S, Conover LF, Bueso-Ramos CE, Kilpivaara O, Stevenson K, Neuberg D, et al. MYBL2 is a sub-haploinsufficient tumor suppressor gene in myeloid malignancy. Elife. 2013;2:e00825.
13. Clarke M, Dumon S, Ward C, Jager R, Freeman S, Dawood B, et al. MYBL2 haploinsufficiency increases susceptibility to age-related haematopoietic neoplasia. Leukemia. 2013;27(3):661-70.
14. Saito C, Hosokawa K, Katagiri T, Kanai A, Matsui H, Inaba T, et al. SLIT1 Mutation in Patients with Acquired Aplastic Anemia: Its Relevance in Immune Pathophysiology Blood, Annual Meeting Abstracts. 2012;120(21):#645.
15. Dunwell TL, Dickinson RE, Stankovic T, Dallol A, Weston V, Austen B, et al. Frequent epigenetic inactivation of the SLIT2 gene in chronic and acute lymphocytic leukemia. Epigenetics. 2009;4(4):265-9.
16. Dasouki MJ, Rafi SK, Olm-Shipman AJ, Wilson NR, Abhyankar S, Ganter B, et al. Exome sequencing reveals a thrombopoietin ligand mutation in a Micronesian family with autosomal recessive aplastic anemia. Blood. 2013;122(20):3440-9.
17. Walne AJ, Dokal A, Plagnol V, Beswick R, Kirwan M, de la Fuente J, et al. Exome sequencing identifies MPL as a causative gene in familial aplastic anemia. Haematologica. 2012;97(4):524-8.