EZH2 mutations and impact on clinical outcome: an analysis in 1,604 patients with newly diagnosed acute myeloid leukemia

The enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase and functional core subunit of the polycomb repressive complex 2 (PRC2), which is a key epigenetic regulator involved in embryonic development and transcriptional repression of genes by catalyzing the methylation of histone H3 at lysine 27 (H3K27me2/3).[1] EZH2 overexpression has been associated with oncogenic activity and worse progression-free survival in several types of cancer including lymphoma, melanoma, and prostate and breast cancer.[2,3] Moreover, the detection of recurrent *EZH2* mutations, both gain-of-function in lymphomas and loss-of-function, e.g. in medulloblastoma, and bladder and renal cancers, point to a mutual role of EZH2 for disease pathology depending on the distinct type of cancer and indicate the potential of EZH2 as a therapeutic target.[4-6] In myeloid malignancies such as myelodysplastic syndromes (MDS), loss of EZH2 activity by inactivating mutations is associated with poor prognosis.[7] More recently, EZH2 inactivation by post translational modification was shown to induce chemoresistance in acute myeloid leukemia (AML).[8] However, data on the frequency and prognostic role of *EZH2* mutations in AML are still scarce and mostly confined to smaller cohorts. To investigate the prevalence and prognostic impact of this alteration in more detail, we analyzed a large cohort of AML patients (n=1,604) for *EZH2* mutations.

All patients had newly diagnosed AML, were registered for trials investigating intensive induction chemotherapy of the Study Alliance Leukemia (SAL) (AML96, AML2003 or AML60+, SORAML), and had available material at diagnosis. All analyses were carried out under the auspices of the SAL-bioregistry. Screening for *EZH2* mutations and associated alterations was performed using Next-Generation Sequencing (NGS) (TruSight Myeloid Sequencing Panel, Illumina) on an Illumina MiSeq-system using bone marrow (BM) or peripheral blood (PB) (*Online Supplementary Appendix*). The myeloid gene panel (Illumina) targets 54 genes associated with myeloid neoplasms including full coding exons of *EZH2*, encoded on the long arm of chromosome 7 (7q36.1). Detection was conducted with a defined cut-off of 5% variant allele frequency (VAF) (median coverage 3,076; range 824-30,565). Patients' clinical characteristics and co-mutations were analyzed according to the mutational status. Furthermore, multivariate analysis was used to identify the impact of *EZH2* mutations on outcome. In addition, a more detailed subgroup analysis of *EZH2*-mut patients was conducted to integrate clinical outcome with the allelic state of mutations, affected functional domains (CXC-SET), and predicted effects of *EZH2* mutations on protein expression.

EZH2 mutations were found in 63 of 1,604 (4%) patients, which is in the range of prevalence (2-13%) typically observed for *EZH2* mutations in myeloid malignancies.[7,9-13] A detailed list of all detected *EZH2* variants is provided in *Online Supplementary Table S1*. In total, 50 of 63 patients (79%) harbored one single mutation in *EZH2*, while 13 individuals carried two different *EZH2* variants (double mutated). Mutations were detected within several exons (2-6; 8-12; 14-20) and functional domains (D1, D2, CXC and SET), respectively (Figure 1A). In line with previous findings, most *EZH2* variants were detected in exons 17 and 18 (28%), comprising the highly conserved SET domain, important for the catalytic activity of the EZH2 protein.[7] The majority of detected mutations (67% missense and 33% nonsense/frameshift) were single nucleotide variants (SNV) (86%), followed by small indel mutations. All frameshift and nonsense mutations resulting in a premature stop of transcription were predicted to be "inactivating" for EZH2 protein expression (Figure 1B). One patient harbored a known *EZH2* gain-of-function variant at position A682 (A677) recurrently found in large B-cell and non-Hodgkin lymphomas.[4] These contrasting observations support recent findings that point to a potential stage-specific role of *EZH2* in AML, exerting a tumor suppressor function during early AML induction and an oncogenic function during tumor maintenance.[12] Other pathogenic SNV found in two or more patients were detected at residues R25 (COSM53003), G159 (COSM96480), R288 (COSM1000721), R313 (COSM6916439), R502 (COSM87274), N673 (COSM87276), R690 (COSM52980), and E745 (COSM1087033) (highlighted in Figure 1A). The p.Glu745Lys mutation (detected in 2 patients) was previously associated with acute lymphoblastic leukemia and lymphoma in Weaver Syndrome patients.[14] Mutations were detected with a median allele burden of 42% (range 6-97%) (Figure 1B). The majority of patients (n=39) were heterozygous for their *EZH2* variant, likely representing clonal events. Subclonal *EZH2* variants (with significantly lower allelic ratio compared to all other co-mutated driver genes) were detected in nine patients. Patients with *EZH2* allelic ratios >70% (n=15) were considered homozygous. *EZH2* mutations were previously detected in both monoallelic and biallelic states in MDS/myeloproliferative neoplasms (MPN).[7]

Descriptive statistics of clinical parameters and associ-

Table 1. Patients' characteristics.

Parameter	*EZH2*-wt	*EZH2*-mut	*P*
N. of patients (n)	1,541	63	
Age, median (years)	56	59	0.044
Disease status, n (%)			0.036
de novo	1,275 (84)	45 (71)	
tAML	63 (4)	5 (8)	
sAML	185 (12)	13 (21)	
WBC, median (Gpt/l)	19.7	15.5	0.429
BM blasts, median (%)	63.4	49.5	0.013
PB blast, median (%)	41.0	27.5	0.043
Karyotype, n (%)			
Normal	796 (55)	30 (53)	0.809
Complex	159 (12)	2 (4)	0.118
ELN risk 2017, n (%)			0.454
Favorable	613 (42)	21 (35)	
Intermediate	412 (28)	17 (28)	
Adverse	435 (30)	22 (37)	
Monosomy 7, n (%)	74 (5)	6 (11)	0.139
Deletion 7q, n (%)	47 (3)	0 (0)	0.319
OS, median (months)	17.1	18.4	0.801
RFS, median (months)	17.4	24.7	0.738

wt: wild-type; -mut: mutations; tAML: therapy-related acute myeloid leukemia; sAML: secondary acute myeloid leukemia; WBC: white blood cells; BM: bone marrow; PB: peripheral blood: ELN: European LeukemiaNET; OS: overall survival; RFS: relapse free survival; N/n: number.

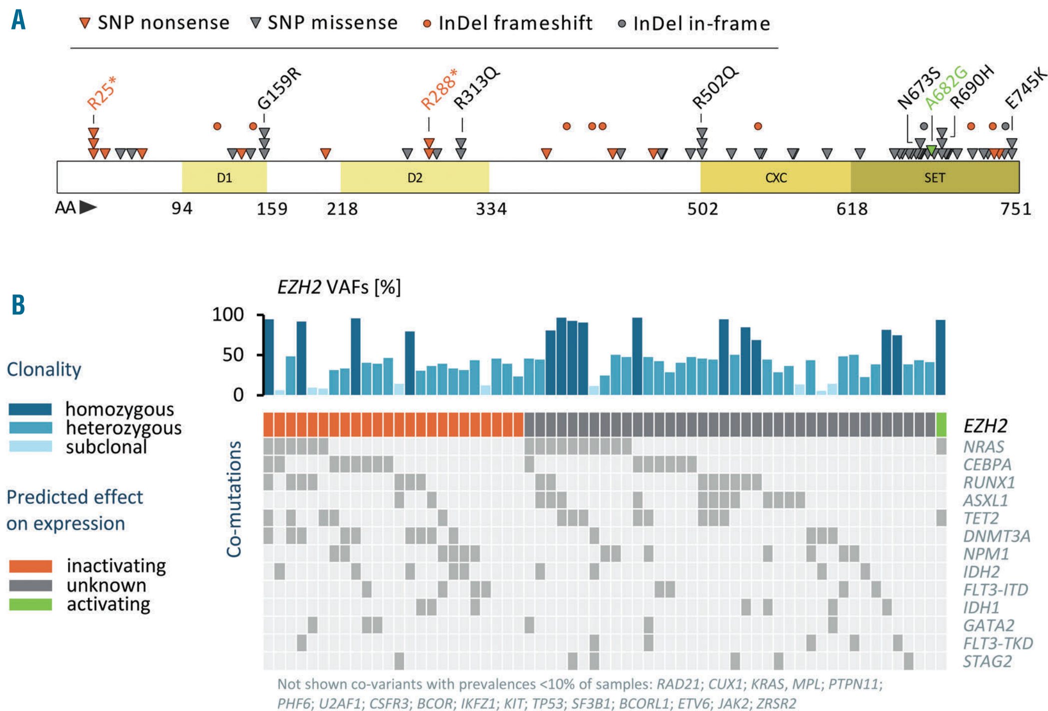

Figure 1. Detected *EZH2* mutations. (A) Schematic illustration showing the position of acquired *EZH2* mutations and the domain structure of *EZH2* (ENST00000320356): D1 (domain I), D2 (domain II), CXC (cysteine-rich domain), and SET (suppressor of variegation-enhancer of zeste-trithorax domain). Recurrent alterations are indicated. (B) *EZH2* variant allele frequencies (VAF) predicted effect on protein expression and associated co-mutations. Clonality was determined by comparing VAF of *EZH2* mutations and co-mutated somatic driver variants. Mutations with uncertain effect on EZH2 protein expression were classified "unknown" (gray). All *EZH2* loss-of-function mutations (nonsense SNPs and frameshift INDEL variants) were considered "inactivating" (orange). One patient harbored a p.Ala682Gly (A677) *EZH2* variant known to be "activating" for protein expression (green).

ated co-mutations revealed significant differences between *EZH2*-mut and wild-type (-wt) patients (Table 1). At diagnosis, patients with *EZH2* mutations were significantly older (median age 59 years) than *EZH2*-wt patients (median age 56 years) (*P*=0.044). In addition, significantly fewer *EZH2*-mut patients (71%) were diagnosed with *de novo* AML compared to *EZH2*-wt patients (84%) (*P*=0.036). Accordingly, *EZH2*-mut patients had a higher rate of secondary AML (sAML) (21%), evolving from prior MDS or after prior chemotherapy (therapy-related AML, tAML) (8%; *P*=0.036). BM (and PB) blast counts differed between the two groups (*EZH2*-mut patients had significantly lower BM and PB blast counts; *P*=0.013), confirming previous reports.[13] In contrast, no differences were observed for white blood cell (WBC) counts at diagnosis, karyotype, Eastern Cooperative Oncology Group (ECOG) performance status and European LeukemiaNet (ELN) 2017 risk category compared to *EZH2*-wt patients. Based on cytogenetics according to the 2017 ELN criteria, 35% of *EZH2*-mut patients were categorized with favorable risk, 28% had intermediate and 37% adverse risk. Initial studies frequently observed associations of *EZH2* mutations in *de novo* AML with monosomy 7 and deletion 7q (-7/7q-).[13] We did not find a significant correlation of the *EZH2* mutational status with -7/7q, confirming other recent reports.[15] However, as expected, patients with *EZH2* allelic ratios >70% (n=15) (considered homozygous) were significantly more often affected by monosomy 7 (27%) compared to patients with heterozygous variants (4.7%) (*P*=0.002). In addition, short tandem repeat analysis of homozygous *EZH2*-mut patients without -7/7q showed that other chromosome 7 aberrations such as monoallelic 7q36.1 microdeletions and/or uniparental disomy may be the cause of EZH2 loss of heterozygosity (*Online Supplementary Appendix*).[7,9] In the group of *EZH2*-mut AML patients, significantly higher rates of co-mutations were detected in *RUNX1* (25%), *ASXL1* (22%), and *NRAS* (25%) compared to *EZH2*-wt patients (with 10%, 8% and 15%, respectively) (Figure 1B and *Online Supplementary Table S2*). Vice versa, concomitant mutations in *NPM1* were (non-significantly) more common in *EZH2*-wt patients (33%) *versus* *EZH2*-mut patients (21%). For other frequently mutated genes in AML, there was no major difference between *EZH2*-mut and -wt patients, e.g. $FLT3^{ITD}$ (13%), $FLT3^{TKD}$ (10%), $CEBPA^{dm}$ (11%), and $CEBPA^{sm}$ (24%), as well as genes encoding epigenetic modifiers, namely, *DNMT3A* (21%), *IDH1/2* (11/14%), and *TET2* (21%). An association of mutations in chromatin modifiers such as *EZH2* and *ASXL1* with mutations in spliceosome or transcription factors like *RUNX1* corresponds to a distinct molecular cluster of co-occurring mutations frequently detected in high-risk MDS.[10,11] This is in agreement with the relatively high rate of secondary AML in *EZH2*-mut patients in our cohort.

In univariate analyses, the *EZH2* mutational status was not associated with the rate of complete remission (CR), relapse-free survival (RFS), or overall survival (OS), with a median OS of 18.4 and 17.1 months for *EZH2*-mut and –wt patients, respectively (Table 1 and Figure 2A) irrespective of the ELN risk group analyzed. Likewise, multivariate analysis with clinical variables such as age, cytogenetics and WBC using Cox proportional hazard regression revealed that *EZH2* mutations were not an inde-

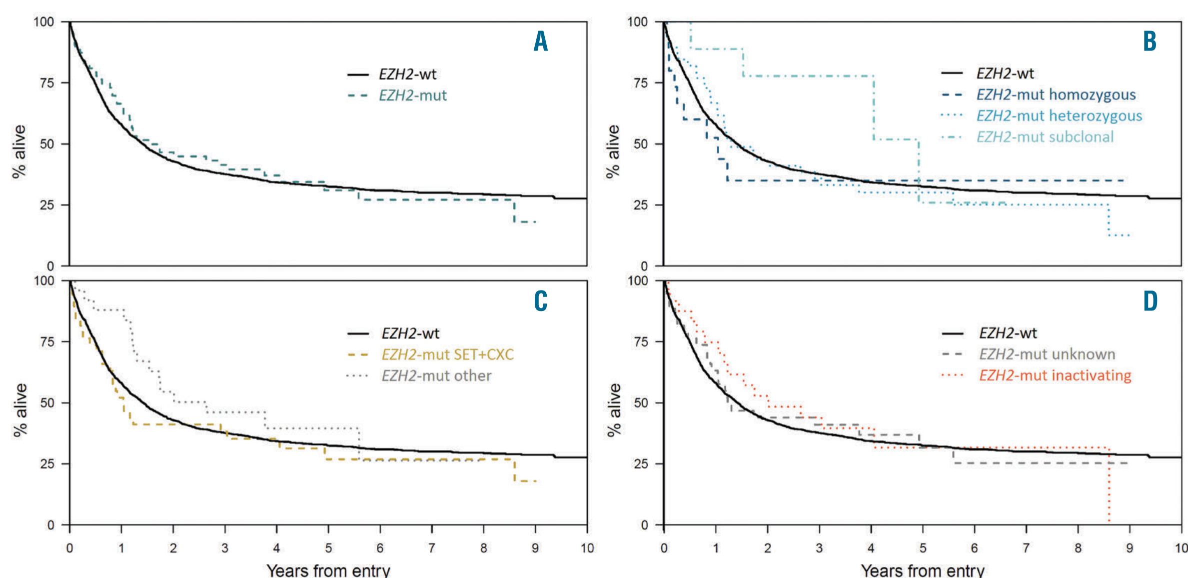

Figure 2. Correlation of *EZH2* mutational status with clinical outcome. Kaplan-Meier analysis showing overall survival of patients with (A) *EZH2* -mutations (-mut) (n=63) *versus* wild type (–wt) (n=1,541) acute myeloid leukemia (B) homozygous (n=15) *versus* heterozygous (n=39) *versus* subclonal (n=9) *EZH2* -mut (C) *EZH2* mutations in affected functional (CXC-SET) (n=38) domains *versus* other EZH2 regions (n=25) and (D) *EZH2* loss-of-function mutations (nonsense/frameshift) (n=24) *versus* missense mutations with unknown functional consequences (n=38).

pendent prognostic factor for OS or RFS. To address the impact of specific *EZH2* mutations on clinical outcome in more detail, survival was next analyzed related to the allelic state (monoallelic *vs.* biallelic) of detected *EZH2* variants, the affected functional domains (CXC-SET) and the predicted effect on the catalytic function of the EZH2 protein. There was a trend towards shorter median OS (12.55 *vs.* 15.61 months) and RFS (8.15 *vs.* 17.29 months) for patients with homozygous mutations compared to individuals with heterozygous (and subclonal) *EZH2* variants, pointing to a potential prognostic impact of high allelic ratio *EZH2* mutations in AML (Figure 2B). Likewise, inferior survival was previously associated with the presence of homozygous *EZH2* mutations in MDS/MPN.[7] Similarly, a slight but statistically not significant effect on OS was observed for patients with mutations in the catalytically active CXC-SET domains (12.4 months) *versus* patients with variants in other less conserved EZH2 regions (31.7 months), demonstrating an association of the affected functional domain with clinical outcome (Figure 2C). However, in all subgroups, no significant association with survival was observed compared to *EZH2*-wt patients. Interestingly, also no significant correlation with clinical outcome was observed for patients with *EZH2* loss-of-function mutations (nonsense/frameshift) (Figure 2D), which are associated with poor prognosis in MDS/MPN.[7] This indicates the importance of other pathogenic mechanisms affecting the epigenetic function of *EZH2* in AML, such as the presence or absence of co-occuring driver mutations and/or post-translational modifications of the EZH2 protein.[8,11] A more stratified assessment of individual *EZH2* variants, patterns of associated co-mutation and functional consequences is warranted to fully understand the prognostic effect of *EZH2* mutations in AML. Thus, taken together, *EZH2* mutations are recurrent alterations in patients with AML. The association with certain clinical factors and typical mutations such as *RUNX1* and *ASXL1* points to the fact that *EZH2* mutations are associated with sAML. However, in contrast to MDS, where *EZH2* mutations are associated with poor prognosis, our data do not indicate that *EZH2* mutations represent an independent prognostic factor in AML.

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