

Relapse of acute myeloid leukemia after allogeneic stem cell transplantation is associated with gain of *WT1* alterations and high mutation load

In relapsed acute myeloid leukemia (AML), the only chance for cure is allogeneic stem cell transplantation (allo-SCT) requiring a suitable donor as well as medical fitness of the recipient.¹ Although in the majority of transplanted AML patients, the donor immune system engrafts successfully, disease relapse after allo-SCT is the most common treatment failure occurring in almost half of patients.² Relapse after allo-SCT may be caused by an immune escape of the AML cells after successful engraftment as well as by the patient's immune system bypassing the immunosuppression and thus counteracting the donor immune cells. While the genetic profile of relapsed AML after chemotherapy largely matches that of AML at initial diagnosis with rather few changes occurring during chemotherapy,³ little is known about the genetic profile of relapsed AML after allo-SCT. Both the conditioning regimen and the graft-versus-leukemia effect may alter the genetic landscape of transplanted AML. Ultimately, the molecular mechanisms leading either to graft failure or immune escape of AML cells still remain unclear. Recently, a study by Quek *et al.* described mutations in AML patients undergoing allo-SCT, however, it was limited to positions of known variants only and did not include a longitudinal analysis of AML patients.⁴ In order to unravel the clonal evolution of relapsed AML after allo-SCT, we performed exome sequencing of matched samples from 12 AML patients, collected at multiple timepoints from initial diagnosis to relapse after allo-SCT. Despite the relatively small cohort, the present study is the most comprehensive genetic analysis of AML

relapse after allo-SCT carried out thus far, with exome sequencing of a total of 53 unique samples. The genetic analysis in the transplant setting is resource intensive and technically challenging, since it requires germline controls both from the donor and the recipient.

A total of seven AML patients (UPN P01-P07) were sequenced at the following five time points: initial diagnosis (Dx), complete remission after chemotherapy (CR-CT), relapse after chemotherapy (Rel-CT), complete remission after allo-SCT (CR-SCT), and relapse after allo-SCT (Rel-SCT) (Figure 1A). Furthermore, we selected another five patients that had already been transplanted in CR-CT and sequenced the specimen at the time points Dx, CR-CT, CR-SCT, and Rel-SCT (UPN P08-P12) (Figure 1B). Samples at CR-CT were used as germline control and available for all patients (n=12), samples at CR-SCT were used to exclude donor germline variants (available for 10 patients). On average, 47.6 million reads were sequenced per sample, the mean coverage of the target region was 99.6x (range 66.5x to 130.1x). At least 94.4% of the target region was covered 10x or higher. Detailed sequencing metrics are summarized in *Online Supplementary Table S1*. Sample preparation and data analysis were performed as described previously⁵ with a minimum variant allele frequency (VAF) of 10% and a minimum of five supporting reads. Variants with VAF at CR-CT <2% were called somatic, variants with VAF at CR-CT ≥2% were called persistent if detected in genes known to be cancer drivers. At the time point of Rel-SCT, somatic variants were detected using CR-CT and CR-SCT samples as controls (Figure 1). As CR-SCT samples were not available for patients P05 and P08, we estimated the donor cell fraction after SCT in these two patients by VAFs of obvious donor germline variants, and subsequently excluded Rel-SCT specific variants with a VAF in

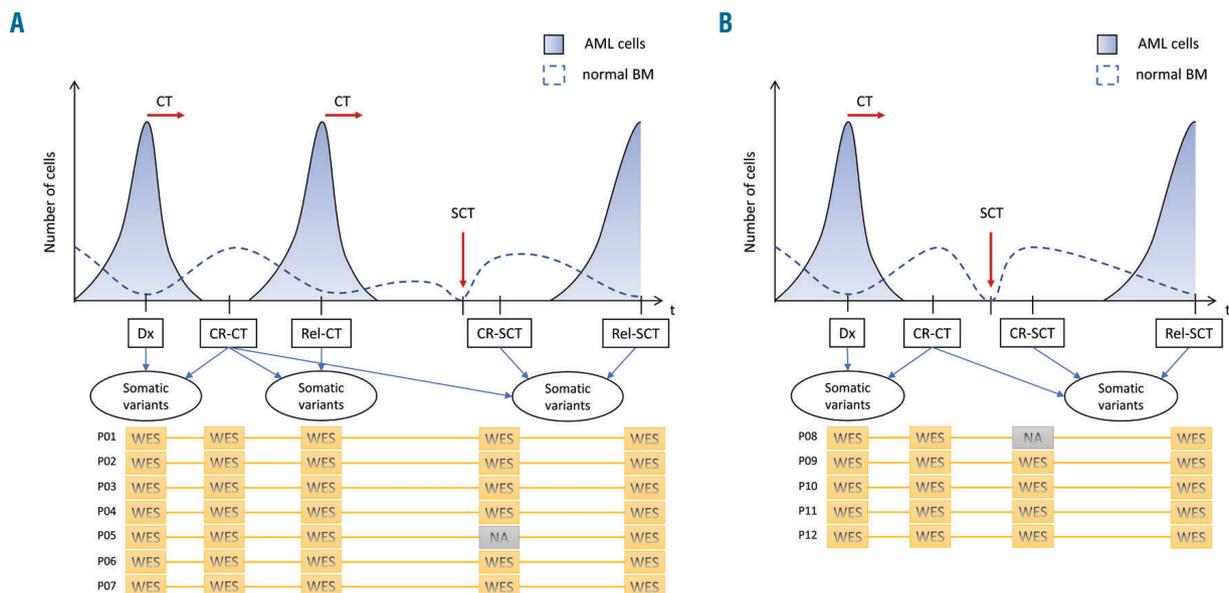


Figure 1. Schematic view of study design and the abundance of AML cells and normal bone marrow cells, respectively. Somatic variants were called with respect to the corresponding remission sample(s). Corresponding patient samples are illustrated below, yellow boxes labelled with WES represent samples that were sequenced, gray boxes represent samples that could not be sequenced. (A) Seven patients had a first relapse after chemotherapy, followed by a second CR and subsequent transplantation. (B) Five patients who were transplanted already in first CR. SCT: stem cell transplantation; CT: chemotherapy; AML: acute myeloid leukemia; CR-CT: complete remission after chemotherapy; CR-SCT: complete remission after SCT; BM: bone marrow; Dx: diagnosis; Rel-CT: relapse after chemotherapy; Rel-SCT: relapse after stem cell transplantation.

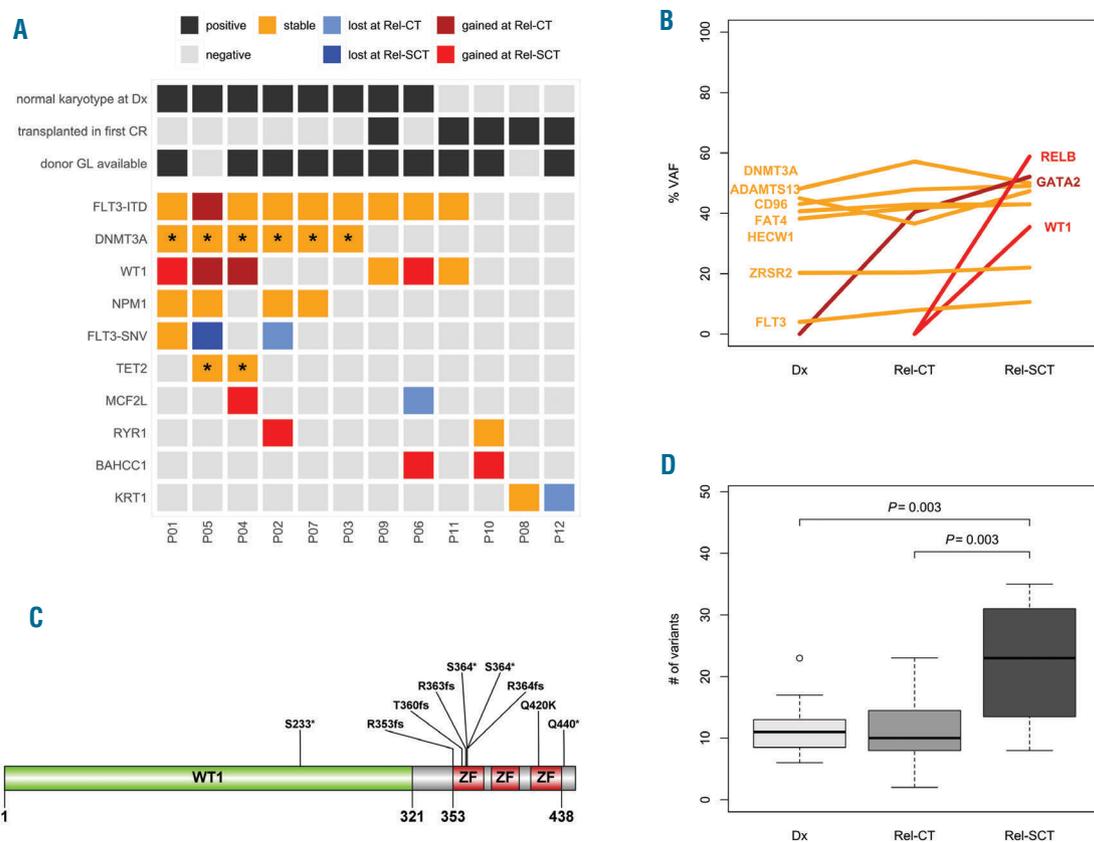


Figure 2. Somatic mutations in AML relapse after allogeneic SCT. (A) Recurrent mutations detected in patients, each column represents one patient. Each row represents the status of a gene or property indicated on the left. The existence of variants at an individual time point is indicated using the color code at the top. Persistent variants detectable at CR-CT samples are marked with an asterisk (*). (B) Exemplary clonal evolution of UPN P01. Individual mutations are displayed by a line connecting VAFs at indicated time points, colored according to their evolutionary pattern. Orange: stable mutation; dark red: mutation gained at Rel-CT and stable at Rel-SCT; light red: mutation gained at Rel-SCT. (C) Domain graph of mutations in *WT1* (NM_000378.4). Amino acid positions and protein changes are presented at the top, domain positions are shown at the bottom. The graph was generated using IBS software, with domain information from PFAM database. An asterisk (*) indicates an introduced stop codon; fs indicates a frame shift. (D) Mutation load at individual time points. Dx: diagnosis; CR: complete remission; GL: germline control sample. Rel-CT: relapse after chemotherapy; Rel-SCT: relapse after stem cell transplantation; VAF: variant allele frequency; ZF: zinc finger.

the range of putative donor germline variants (*Online Supplementary Figure S1*). The criteria for donor germline variants were defined as: (I) presence at Rel-SCT only (VAF=0% in all previous samples), (II) annotation as common single nucleotide polymorphisms (SNPs) in the dbSNP database (minor allele frequency (MAF) $\geq 1\%$), and (III) minimum coverage of 100x in order to increase the reliability of cell fraction estimation by VAFs. Thereby, we detected >500 donor germline variants per sample (*Online Supplementary Figure S1A*). Rel-SCT specific variants not matching these criteria but with a VAF in the range of donor germline variants were excluded from somatic variant analysis in the two patients without an appropriate control (P05: 10%-26.7%, P08: 25.5%-100%) (*Online Supplementary Figure S1B*). While somatic variants in leukemia subclones might be missed, this approach dramatically reduces the false positive rate, as donor germline variants cannot be confounded with Rel-SCT specific somatic variants. *Online Supplementary Figure S1C,D* show the robustness of this approach, as we applied our method to CR-SCT samples with a known donor cell fraction of 100%. Finally, all somatic variants underwent exclusion of sequencing artifacts by an in-house blacklist of error-prone regions and manual alignment inspection in order to reduce false positive variant

calls. *FLT3*-ITD and *NPM1* mutation status was obtained from routine assays.

The median age of the patients was 47.5 (range 21-73), most patients (8/12) were cytogenetically normal (CN) at Dx (Figure 2A, *Online Supplementary Table S2*). Aberrant cytogenetics at Dx included t(9;11) (n=1), trisomy 8 (n=1) and complex karyotypes (n=2). In our cohort, a median of 26 somatic variants were detected by next-generation sequencing at any time point per patient (range 10-39) with a total of nine genes recurrently mutated (Figure 2A, *Online Supplementary Figure S2*, *Online Supplementary Table S3*). Consistent with a high risk of relapse, *FLT3*-ITD was the most common mutation (n=9), followed by mutations in *DNMT3A*, *WT1*, and *NPM1* (n=6, n=6, n=4, respectively). Mutations in *DNMT3A* and *NPM1* were stable at all time points with 6/6 *DNMT3A* mutations showing persistence at CR-CT. Only two patients had mutations in *WT1* at Dx, corresponding to the previously reported overall prevalence of *WT1* mutations in diagnostic AML samples of 6-13%.^{3,6,7} Interestingly, *WT1* mutations were recurrently gained during the course of the disease (n=4) resulting in a substantially higher number of *WT1* mutations at Rel-SCT (6/12 patients). In order to determine if gained *WT1* mutations were acquired *de novo* during therapy, or already present at subclonal levels

at earlier time points, we evaluated targeted amplicon sequencing data with a minimum coverage of 595x at *WT1* mutation positions. We identified two subclonal mutations in only one out of four patients with gained *WT1* mutations (VAF <1% each) at relapse prior to SCT, suggesting a subclone harboring both mutations (UPN P06, p.Q420K and p.S364*). Recurrent mutation gains were also detected in *BAHCC1* and *RYR1* (n=2 each), whereas a recurrent loss was observed only for *FLT3* point mutations, both located in the C-terminal tyrosine kinase domain (D835K, n=2), while another *FLT3* point mutation (F590N, UPN P01) was stable throughout the follow-up. In general, the majority of mutations detected at Dx were also found at Rel-CT and Rel-SCT (120/137, 88%), however, individual mutations were lost or gained during the course of the disease (Figure 2B, *Online Supplementary Figure S2* and *Figure S3*). Of note, all *WT1* mutated patients harbor at least one mutation in the hotspot region of exon 7, introducing a premature stop codon (Figure 2C, *Online Supplementary Table S3*). Overall, the number of somatic variants was similar at Dx (median 11, range 6-23) and Rel-CT (median 10, range 2-23), while after allo-SCT, the mutation load was significantly higher (median 23, range 8-35, $P=0.003$, two-tailed unpaired Student's *t*-test, Figure 2D, *Online Supplementary Figure S2*). Although we estimated the donor cell fraction and excluded variants likely representing donor germline variants, both patients without CR-SCT samples were excluded from the mutational load analysis, as the genetic burden of Rel-SCT might be underestimated in these patients. In general, the VAF of most Rel-SCT specific mutations suggest that a major clone is harboring these alterations (*Online Supplementary Figure S3*, *Online Supplementary Table S3*).

During the course of AML, patients show a substantial number of stable mutations at multiple time points. Only few changes of the mutational profile can be observed between Dx and Rel-CT. Individual gain or loss of mutations points towards relapse originating from either the dominant AML clone at Dx with few additional alterations, or from a very similar but ancestral subclone present at Dx expanding after chemotherapy. In general, resistance to chemotherapy might be mediated even by a single mutation or only a few genetic alterations which can be found after CR-CT. In a previous study, we showed that *WT1* mutations were gained in relapsed CN-AML patients after CR-CT (14% at Rel-CT vs. 8% at Dx).³ In contrast, after allo-SCT the mutational load in AML is significantly higher with frequent acquisition of *WT1* loss-of-function mutations constituting a potential mechanism of escape from the donor immune system. The high overall mutational load observed at Rel-SCT might be related to the mutagenic effects of conditioning regimens, including both chemotherapy and total body irradiation or an increased need for additional alterations in order to escape the therapy. The observed association of *WT1* mutations with both Rel-CT and Rel-SCT in the present study is consistent with results presented at the recent EHA meeting.⁸ Previous studies have shown that *WT1* mutations are associated with chemotherapy resistance in CN-AML⁹ and gained at relapse after chemotherapy.¹⁰ The study by Quek *et al.* reports a total of five *WT1* mutations at Dx in 49 relapsing patients (10%). Moreover, the authors analyzed paired pre- and post-SCT samples of 29 patients and observed a total of three *WT1* mutations in AML after allo-SCT.⁴ Thus, the prevalence of *WT1* mutations after allo-SCT is strikingly higher in our study (6/12 vs. 3/29, 50% vs. 10%). Further, one of the *WT1* mutations detected at Rel-SCT in that study

was detected only at a subclonal level with a VAF of 7%, which is below our detection cutoff. Quek and colleagues might have missed additional *WT1* mutations as they did not include germline samples in order to detect somatic variants systematically. Instead, they focused on previously reported somatic variants in hematopoietic diseases and considered novel variants only if adjacent to known positions. Moreover, the authors reported only mutations found by two different variant callers, namely GATK and VarScan. Thus, the burden of *WT1* mutations might have been underestimated in that study. In distinct genetic subgroups of AML, *WT1* mutations have been described with varying impact on prognosis.^{11,12} In biallelic *CEBPA* mutant AML, the prognostic relevance remains controversial, while in CN-AML *WT1* mutations associate with shorter event-free survival. *WT1* mutations have been described as rare events in CN-AML and in our cohort, only one out of eight CN-AML patients was *WT1*-mutated at Dx. At Rel-SCT, all *WT1* mutation gains were detected in CN-AML patients. Thus, a total of 5/6 *WT1* mutations were detected in CN-AML patients, pointing towards the aggressiveness of *WT1*-mutated disease in this genetic subgroup. As we used samples at CR-CT as germline control samples, our study may be enriched for CN-AML patients (8/12) who are more likely to achieve a CR. In contrast to *WT1* gene expression as a prognostic marker in pre-allo-SCT AML,^{13,14} little is known about the prognostic value of *WT1* mutations in patients allografted for AML. Quek *et al.* reported that the presence of *WT1* mutations before allo-SCT correlates with increased relapse risk.⁴ Of note, in acute lymphoblastic leukemia *WT1* mediates the graft-versus-leukemia effect after allo-SCT.¹⁵ It is intriguing to speculate that the truncating mutations of *WT1* may disrupt a critical epitope of AML cells which is targeted by the donor immune system. Recurring *BAHCC1* or *RYR1* mutations have not been described in AML before, but recurrent deletions of *BAHCC1* in hepatocellular carcinoma point towards a potential role of this gene in cancer,¹⁶ while *RYR1* was shown to be expressed in a variety of hematopoietic cells¹⁷ and to be associated with immune response and T-cell activation.^{18,19}

Taken together, deciphering the evolution of AML relapse after allo-SCT and the potential role of *WT1* mutations in disease progression are essential to resolve this challenging clinical situation.

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