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Molecular characterization of AML with *RUNX1-RUNX1T1* at diagnosis and relapse reveals net loss of co-mutations

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Abstract

AML with *RUNX1-RUNX1T1* fusion is a WHO entity with a favorable outcome following intensive chemotherapy. The absence of *RUNX1-RUNX1T1* transcripts in remission defines complete molecular response and correlates with a superior survival. However, a significant proportion of patients still relapses and defining molecular risk factors that identify patients at diagnosis or at molecular remission that are at risk of relapse could help tailor treatment strategies for those high risk patients. Here, we analyze a cohort of 94 patients that reach a molecular remission (MR) following intensive treatment and identify 21 patients that relapse despite achieving MR. Using targeted sequencing of 63 genes implicated in hematologic malignancies we show that at diagnosis patients who relapse following MR have a higher burden of co-mutated genes than patients that do not relapse (median = 2 vs median = 0; $P = 0.0156$). This resulted in a relapse free survival rate of 65% vs 86% at 2 years, respectively (≥1 co-mutation vs no co-mutation, $P = 0.02$) with a trend for inferior overall survival (n.s.). Applying sensitive sequencing to reassess mutations at relapse in paired samples of 17/21 patients we demonstrate a net loss of co-mutations at relapse: median 2 (range 0–5) vs 1 (0–4) at diagnosis and relapse ($P = 0.048$). At relapse more patients had no detected co-mutation compared to diagnosis (47% vs 17%, $P = 0.034$). Co-mutations at diagnosis, therefore, might represent a general susceptibility of the AML clone to acquire mutations and the true nature of 2nd hit mutations that drive leukemia has to be defined for AML with *RUNX1-RUNX1T1* fusion.

Introduction

AML with *RUNX1-RUNX1T1* fusion is generally considered a good risk disease. However, about half of the patient's relapse despite achieving a complete remission and the overall survival of those patients is only 51%.[1,2] The absence of *RUNX1-RUNX1T1* transcripts following treatment defines molecular remission (MR) and minimal residual disease (MRD) negativity confers excellent prognosis.[3,4] Risk stratification according to MRD is possible and initial studies allocating MRD positive patients to allogeneic stem cell transplantation have been undertaken.[5] In light of this data it is worth noting that despite achieving MR about 10% to 30% of patients relapse.[4,6,7] In our recent report, we have identified 16% of patients who relapse despite achieving a complete molecular response.[8] The definition of high-risk disease at diagnosis or at molecular remission is therefore desirable. Several reports have addressed the pattern of co-mutations with *RUNX1-RUNX1T1* at diagnosis and identified recurrent mutations in known driver genes.[9,10] We reanalyzed all 94 patients who had a molecular remission following intensive treatment to assess the implication of the number of co-mutations at diagnosis. The twenty-one out of ninety-four patients relapsed and paired samples at diagnosis and relapse were available for 17 of those 21 patients. To gain better insight into relapse mechanisms and the understanding of clonal evolution that drives relapse we sequenced those paired samples in patients that relapsed despite achieving a MR.

Patients, materials and methods

Patients and MRD detection

All patients gave their written informed consent for scientific evaluations. The study was approved by the Internal Review Board and adhered to the tenets of the *Declaration of Helsinki*. We reanalyzed 94 patients that were diagnosed between 2005 und 2017 with AML with *RUNX1-RUNX1T1* fusion and

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Table 1

Patient characteristics.

Patient characteristics	All patients (n = 94)	Relapse (n = 21)	No relapse (n = 73)	*P* value
Age				
Median (range)	50 (18–83)	50 (18–71)	50 (18–83)	0.796
Sex				
Male (%)	39 (41)	7 (33)	32 (44)	0.395
Female (%)	55 (59)	14 (67)	41 (56)	
Morphologic diagnosis				
AML with minimal differentiation n (%)	1 (1)	1 (5)	0	0.061
AML without maturation	20 (21)	5 (24)	15 (21)	0.826
AML with maturation	48 (51)	10 (48)	38 (52)	0.677
AML not specified	25 (27)	5 (24)	20 (27)	
White blood cell count (10^9/l)				
Median	7.1	9.9	6.9	0.29
Range	1.5–119	2.3–119	1.5–91	
Hemoglobin (mg/dl)				
Median	9	8	9	0.139
Range	3–19	4–19	3–15	
Platelet count (10^9/l)				
Median	34	28	40	0.112
Range	5–247	6–70	5–247	
Blast count in BM (%)				
Median	56	64	54	0.331
Range	9–96	16–90	9–96	

achieved MR following intensive treatment (patients with complete molecular remission (CMR) and MRD low in our previous report,[8] detailed patient characteristics see Table 1). We applied quantitative real-time PCR to detect *RUNX1-RUNX1T1/ABL1* ratios with a sensitivity of 0.001%. Molecular remission (MR) was defined as qPCR ratio of 0. Relapse was defined as a 3 log-fold increase in *RUNX1-RUNX1T1/ABL1* ratio above the last MR value and an absolute ratio above 1 (all 21 patients) and/or overt hematological relapse (4 patients had evidence of relapse as evaluated by central morphologic review of bone marrow slides). We identified 21 patients with relapse. For those patients we have analyzed a total of 171 bone marrow specimen by qPCR in the course of disease. In the median each patient received 7 bone marrow analyses (range 4–19). The median interval between each bone marrow analysis was 2.4 months (range 0.2–65). Characteristic findings at relapse are given in Supplemental Table 1 (Supplemental Digital Content, http://links.lww.com/HS/A24). Relapse free survival (RFS) was defined as time from diagnosis to detection of molecular or overt relapse. Overall survival (OS) was defined as time from diagnosis to death of any cause.

Next generation sequencing

All samples were analyzed by a gene panel containing *ASXL1, ASXL2, ATM, BCL2, BCOR, BCORL1, BIRC3, BRAF, BTK, CALR, CBL, CSF3R, CSNK1A1, CXCR4, DNMT3A, EGR2, ETNK1, ETV6, EZH2, FBXW7, FLT3, FOXO1, GATA1, GATA2, ID3, IDH1, IDH2, JAK2, KIT, KLF2, KRAS, MAP2K1, MPL, MYC, MYD88, NF1, NFKBIE, NOTCH1, NOTCH2, NPM1, NRAS, PHF6, PIGA, PLCG2, POT1, PTPN11, RAD21, RUNX1, SAMHD1, SETBP1, SF3B1, SRSF2, STAG2, STAT3, STAT5B, TCF3, TET2, TP53, U2AF1, UBR5, WT1, XPO1*, and *ZRSR2*. The library of 63 genes was generated with a TruSeq Custom Amplicon Low Input Kit (Illumina, San Diego, CA), following the manufacturers' protocol. The library contained molecular tags (unique molecular identifiers (UMI) that allow the detection and quantification of the individual molecule of each template DNA fragment. This tag was incorporated and sequenced, enabling the accurate detection of true variants with high resolution up to 1% VAF as PCR duplicates can be identified and discarded. The library was sequenced and demultiplexed on a Nextseq instrument (Illumina, San Diego, CA) as described previously.[11] The FASTQ files were further processed using the Sequence Pilot software version 4.3.1 Build 502 (JSI Medical Systems, Ettenheim, Germany) for alignment and variant calling. Analysis parameters were set according to manufacturers' default recommendation. Validity of the somatic mutations was checked against the publicly accessible COSMIC v78 (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic) and ClinVar database. Functional interpretation was performed using SIFT 1.03 (http://sift.jcvi.org) and PolyPhen 2.0 (http://genetics.bwh.harvard.edu/pph2).[12] Additionally, TP53 variants were verified using the IARC repository.[13] Single-nucleotide polymorphisms (SNP) were annotated according to the NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp; Build 147) and ExAC population frequency database. Variants of uncertain significance were excluded from statistical analyses. FLT3-ITD was analyzed by gene scan. We used 25 to 50 ng of genomic DNA for each sequencing reaction. The mean coverage of the reported mutations at diagnosis was 4654 (range 667–13,011), the mean coverage at relapse was 3243 (537–13,269). At diagnosis the wild type allele was covered with a mean of 3468 reads (658–7573) and the mutated allele with a mean coverage of 1376 reads (9–6813). At relapse the wild type allele was covered with a mean of 2716 reads (range 537–7715) and the mutated allele with a mean coverage of 1183 reads (42–5554). The detected mutation, the mutation type, the wild type read count and the mutational read count are provided in Supplemental Table 2 (Supplemental Digital Content, http://links.lww.com/HS/A24).

Statistical analysis

Statistical analysis was performed using Prism 7.03 software by GraphPad (La Jolla, USA). Survival differences were calculated by log-rank test and for group comparisons the Mann-Whitney test was utilized. The paired *t* test was used for the analysis of paired results. Survival fractions were computed using the Kaplan-Meier method, confidence intervals or survival percentages were computed using the log-log transform method.

Results

We recently identified 94 patients that were diagnosed between 2005 and 2017 with AML with *RUNX1-RUNX1T1* fusion and achieved MR following intensive treatment.[8] The twenty-one out of ninety-four patients relapsed despite this good risk situation. There was no significant difference in clinical parameters upfront in patients who relapsed following MR compared to patients who did not relapse (Table 1). We have previously reported that the mutational landscape at first diagnosis of patients who achieve MR compared to those who do not is comparable. Only the presence of *ASXL1* mutations was associated with shorter relapse free survival (RFS).[8] We now reanalyzed the cohort and compared the number of co-mutated genes with *RUNX1-RUNX1T1* at diagnosis in patients who relapsed following MR (21/21 diagnostic samples available for sequencing) with patients without relapse (42/73 diagnostic samples available for sequencing). We here show for the first time that a higher total number of co-mutated genes at diagnosis was significantly associated with relapse (median = 2 (range 0–3) vs median = 0 (0–4); $P = 0.0156$, Fig. 1A). And significantly more patients with relapse following MR had additional mutations accompanying *RUNX1-RUNX1T1* at diagnosis: 19/42 (45%) of patients who did not relapse had an additional mutation vs 16/21 (76%) of patients that relapsed ($P = 0.02$). The variant allele frequency of all mutations detected at diagnosis was not different in the 2 groups (Fig. 1B). The median relapse free survival of patients without detectable co-mutated genes was not reached and was 41 months for patients with ≥1 co-mutated gene. This resulted in a RFS rate of 86% vs 65% at 2 years, respectively (Fig. 2A, $P = 0.02$). There was a trend for a longer overall survival (OS) in patients without co-mutations than in patients with co-mutations however this was not significant: OS rate at 5 years was 89% versus 69% respectively (n.s., Fig. 2B). Specifically there was no significant difference in overall survival post relapse for all 21 patients when analyzed according to number of co-mutations at diagnosis (Fig. 2C). This could be due to different number of patients receiving salvage chemotherapy and allogeneic transplant. 11/21 (52%) patients received allogeneic transplant and 10/21 (48%) did not. In the cohort with 0, 1 and > 1 mutated genes at diagnosis, 1/4, 3/8, and 7/9 were transplanted for relapsed AML respectively (Fig. 2C lower panel, n.s.).

To better understand how the mutational burden influences AML relapse we investigated the evolution of mutations at relapse. For 17/21 patients who relapsed following MR paired diagnostic and relapse samples were available for sensitive sequencing with the 63 gene panel. An overview of mutations detected at diagnosis and at relapse is given in Figure 3A. At diagnosis we observed a total of 28 somatic mutations in 11 different genes co-occurring with *RUNX1-RUNX1T1* fusion in 14/17 patients: *ASXL1* (n = 6), *ASXL2* (5), *KIT* (5), *NRAS* (2), *CSF3R* (2), *FLT3*-TKD (2), *FLT3*-ITD (1), *IDH2* (1), *KRAS* (1), *TET2* (1), *RAD21* (1), *SRSF2* (1). The median number of mutations per patient was 2 (range 0–5). An overview of the clonal evolution from diagnosis to relapse for each patient including the VAF of all mutations is given in Figure 3B. At relapse we identified a total of 14 mutations in 7 different genes in 9/17 patients (*KIT* (n = 4), *ASXL1* (3), *ASXL2* (2), *TET2* (1), *IDH2* (1), *FLT3*-TKD (1), *FLT3*-ITD (1), *PHF6* (1); *TET2* n.a. in 1 patient). At relapse the median number of mutations per patient was 1 (range 0–2). 17 mutations in 9 genes (*ASXL2, ASXL1, KIT, NRAS, CSF3R, FLT3*-TKD, *KRAS, RAD21, SRSF2*, Fig. 4A) were lost at relapse and 4 mutations in 3 genes (*KIT, PHF6, TET2*) were gained at relapse. The median number of mutations per patient at relapse was significantly reduced compared to diagnosis ($P = 0.0485$, Fig. 4B). Patients lost a median of 1 mutation (range 0–4) and significantly fewer patients had co-mutations with *RUNX1-RUNX1T1* fusion at relapse: 14 patients (82%) had at least 1 co-mutation detected at diagnosis and only 9 patients (53%) at relapse ($P = 0.034$). The median variant allele fraction (VAF) of co-occurring mutations at diagnosis was 23% (range 1–24) and 42% (range 2–67) at

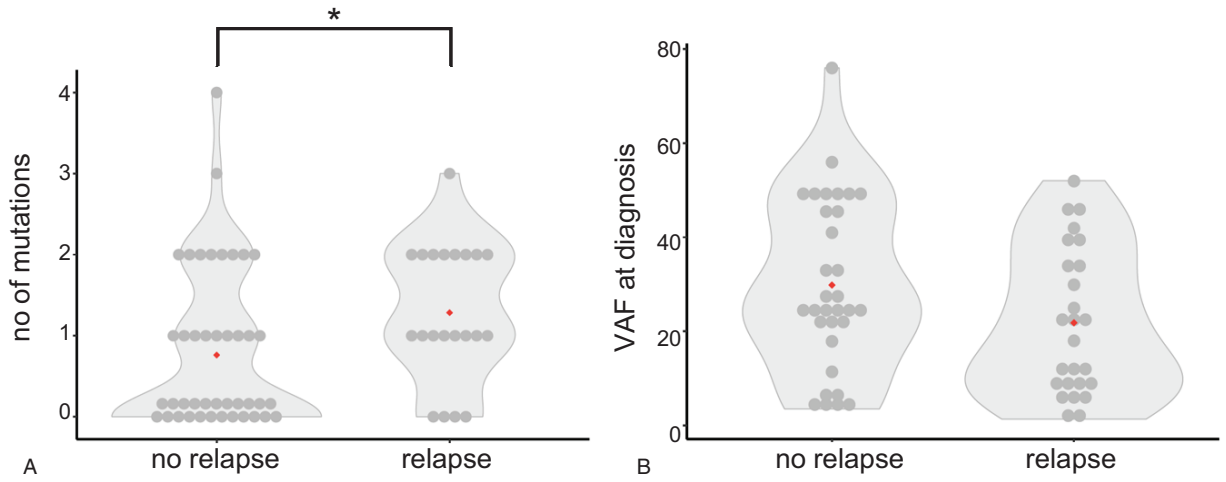
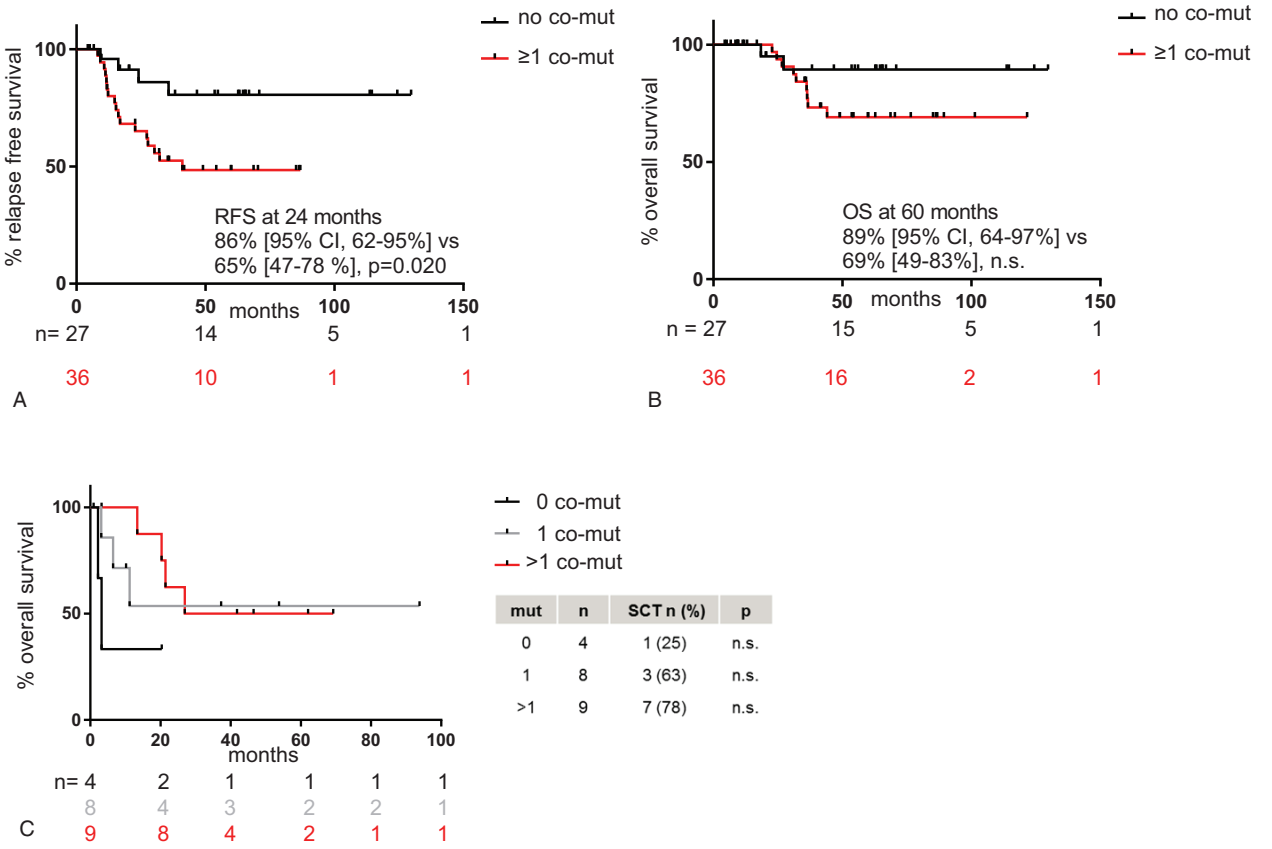


Figure 1. The number of co-mutations with *RUNX1-RUNX1T1* at diagnosis is associated with relapse. A) Samples of 42 patients that never relapsed and 21 patients that relapsed were available for sequencing. The absolute number of mutated genes in patients with relapse and without relapse (red dot, mean) is significantly different ($P = 0.0156$). B) The VAF of mutations of patients that relapse and patients without relapse is shown (n.s., red dot, mean).



mut	n	SCT n (%)	p
0	4	1 (25)	n.s.
1	8	3 (63)	n.s.
>1	9	7 (78)	n.s.

Figure 2. The number of co-mutations is associated with relapse free survival (RFS). A) RFS of patients with no co-mutated gene vs patients with ≥1 co-mutated gene. B) Overall survival (OS) of patients with no co-mutated gene vs patients with ≥1 co-mutated gene. C) Survial of patients post relapse according to number of co-mutated genes. Table: Relatively more patients were transplanted following relapse in the group of patients with more co-mutated genes (n.s.).

relapse (1.9 fold, n.s.). The VAF of mutations that persisted was unchanged (median at diagnosis 47% vs 36% at relapse, n.s., Fig. 4C) but the VAF at diagnosis of mutations that were lost at relapse was significantly lower than the VAF of mutations that persisted at relapse: median 11% (range 1–50%) vs 47% (range 9–50%, $P=0.0148$, Fig. 4D). Analyzing in depth mutations that were lost at relapse revealed that 8/17 mutations that were lost at relapse had an initial VAF of >20% and also mutations with a VAF of 50% at diagnosis were lost at relapse. One reason for a reduced detection of mutations at relapse could be attributable to a lower disease burden at relapse compared to diagnosis. We analyzed disease burden at diagnosis and relapse by comparing the *RUNX1-RUNX1T1/ABL* ratio and show that the ratio was not significantly higher at diagnosis than at relapse (median 73 (range 4–507) vs 37 (2–500), n.s., Fig. 4E). Moreover as only 4 patients had evidence of relapse by centrally evaluated morphology, we show that clonal loss is also observed in patients with morphologic relapse (Fig. 3, indicated with m). To address the impact of mutation gains at relapse we analyzed RFS and OS in the 17 patients with analyzed paired samples: a gain of mutations at relapse was associated with a significantly shorter RFS ($P=0.009$) and a reduced OS (n.s., Fig. 5).

Discussion

In this report, we address the number of co-mutations with *RUNX1-RUNX1T1* at diagnosis and relapse in a cohort of patients who achieve a molecular remission following intensive chemotherapy. We aimed to identify prognostic markers in this group of patients that generally has a very good prognosis.

In the recent years, novel molecular markers for prognosis have emerged in AML with *RUNX1-RUNX1T1*.[14–18] Although *KIT* and *FLT3* mutations were associated with inferior outcome, the significance of these findings is limited due to the retrospective nature of the analysis the data were derived from. In recent reports, only high burden mutations in *KIT* or *FLT3* were associated with an inferior prognosis.[9,19] And what is more, when analyzing MRD and mutational status in the same cohort only MRD remained as prognostic factor in multivariate analysis.[20] In our recent report, we showed that the co-mutation pattern in the whole cohort of patients was similar to that reported recently by others on core binding factor AML with *RUNX1-RUNX1T1* fusion.[8–10] In this report, the best prognostic value had a repetitive and sensitive detection of MRD negativity, but this is a marker that can only be assessed in the course of disease. In our current analysis, we now show that a higher total number of mutations at diagnosis is significantly associated with relapse. This did not translate into a significantly shorter OS of patients with more than 1 co-mutation at diagnosis, which is potentially caused by a skewed frequency of allogeneic transplantation for relapse in the subgroups and the overall low number of patients in each subgroup. Nevertheless, risk of relapse is an important measure in AML as salvage chemotherapy is usually aggressive and transplantation in first CR is advised only in high-risk groups. While the OS of patients following first relapse is generally reduced in AML with *RUNX1-RUNX1T1*

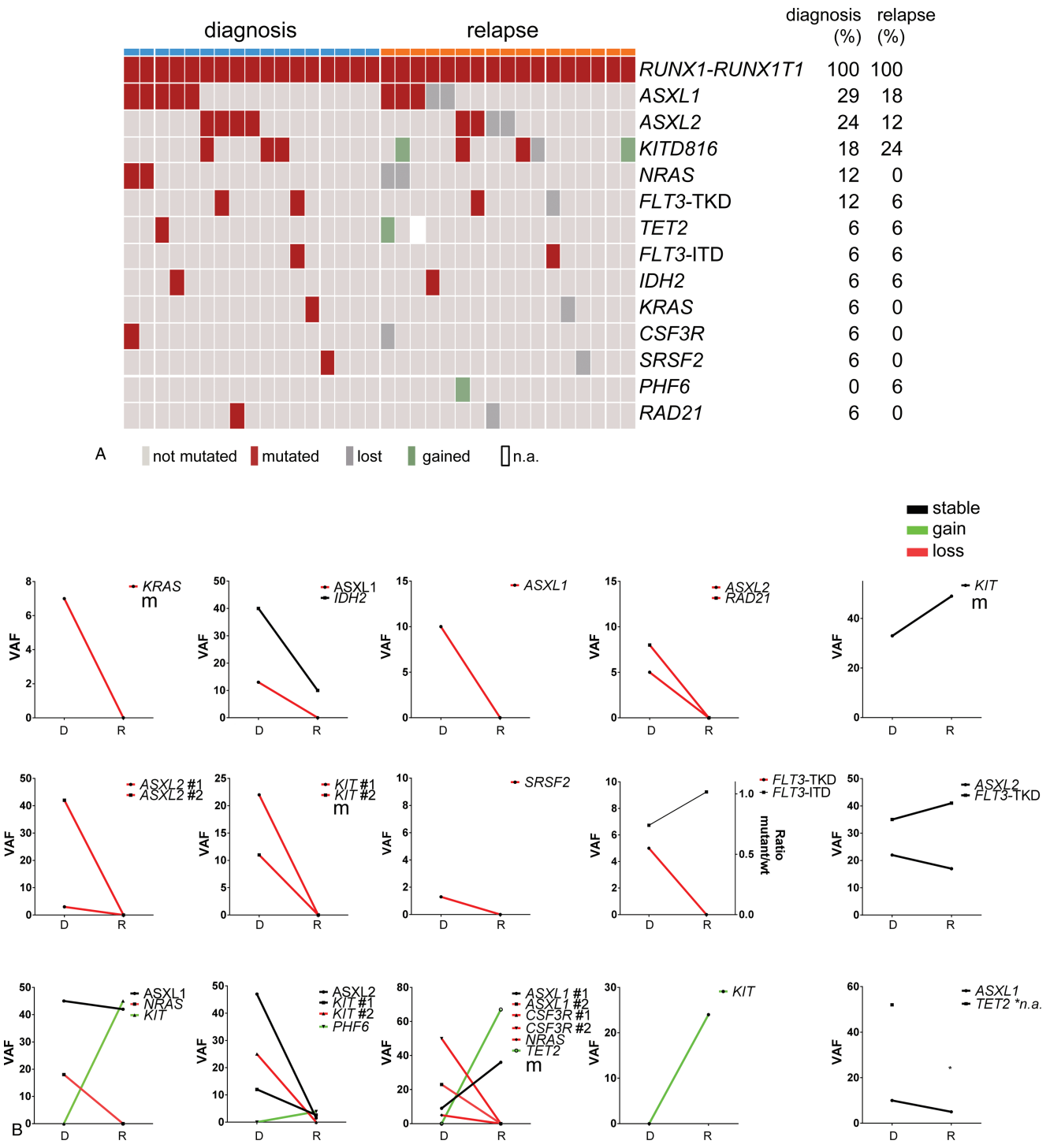


Figure 3. Clonal evolution of paired samples at diagnosis and relapse. For 17/21 patients who relapsed following molecular remission paired samples at diagnosis and relapse were available for sequencing analysis. A) Heatmap of mutated genes at diagnosis and relapse: shown are genes with ≥1 mutation at diagnosis or relapse. B) Patient based mutation tracking: shown is the variant allele frequency (VAF) at diagnosis (D) and at relapse (R) of the indicated gene. Mutations that are lost at relapse are shown in red, mutations that are gained at relapse in green, stable mutations in black. 2 patients had no mutation at diagnosis or relapse and are not shown. For *FLT3*-ITD the mutant/wildtype ratio is given. 4 patients with evidence of relapse in centrally reviewed morphology are indicated with m.

fusion,[2] we have previously shown that patients that relapse despite molecular remission still benefit from allogeneic transplant.[8] The fact that a higher mutational burden is associated with relapse is also of interest from a biologic point of view: One could speculate that a higher mutational burden represents a vulnerability of the AML clone to acquire mutations and therefore also acquire resistance mutations or mutations that render the clone more fit and aggressive. This could be a general principle in AML as we have previously reported that more than 2 additional mutations correlated with shorter OS in *RUNX1* mutated AML.[21] A recent report analyzed the nature of additional co-mutations in core binding factor leukemias and showed that multiple mutations in signaling genes (ie, *FLT3, KIT, NRAS, KRAS*) as part of a clonal interference confer an

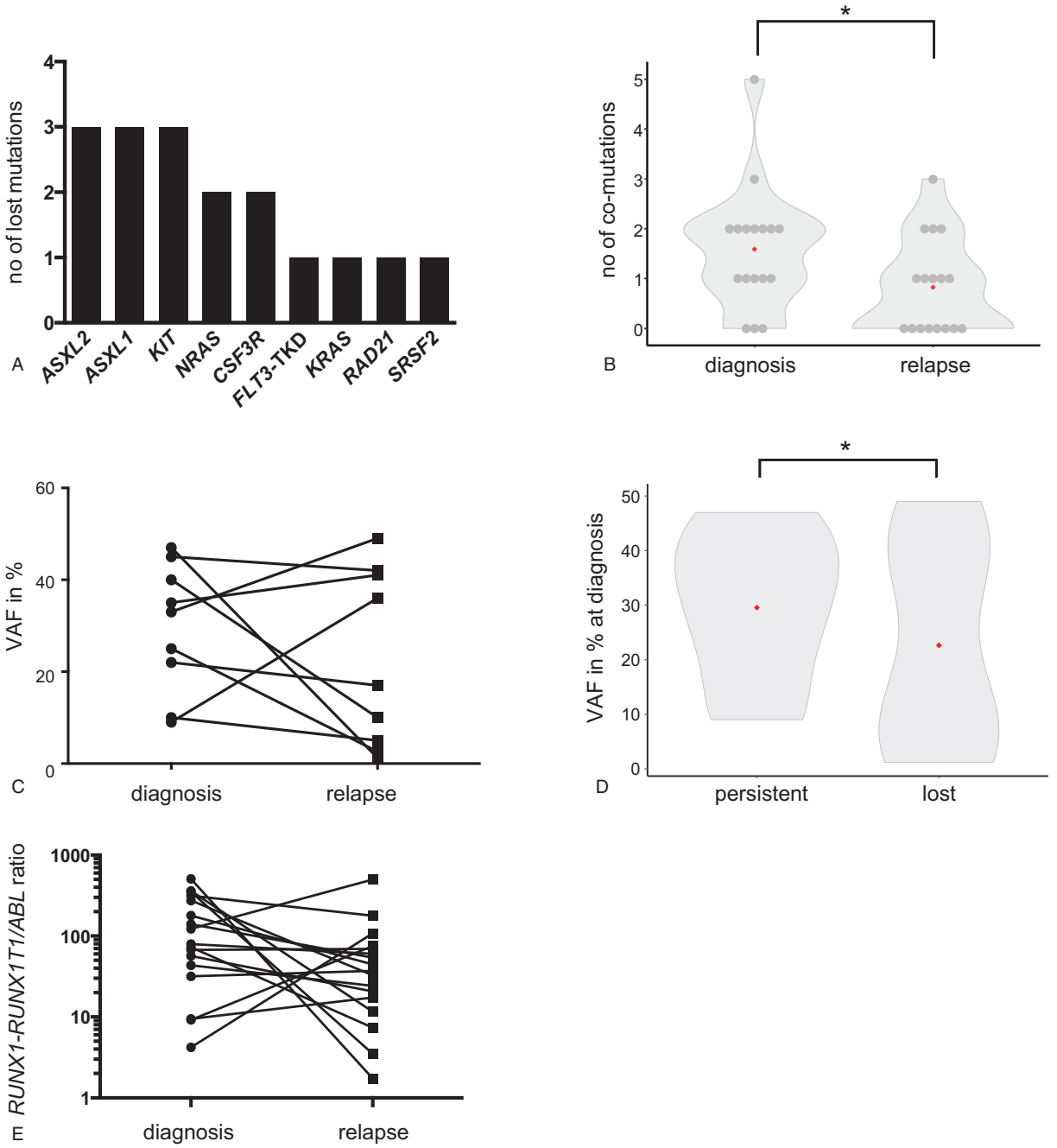


Figure 4. Net loss of co-mutations at relapse. For 17/21 patients who relapsed following MR paired diagnostic and relapse samples were available for sequencing. A) Mutations in known leukemia associated genes are lost at relapse. Shown is the number of mutations in the indicated genes that are lost at relapse. B) The number of mutations at relapse is significantly reduced. Shown is the absolute number of mutations at diagnosis and at relapse ($P=0.0485$), red dot, mean. C) The change in VAF at diagnosis and at relapse of persisting mutations is given (n.s., $P=0.389$). D) The VAF at diagnosis of mutations that persist at relapse and the VAF at diagnosis of mutations that are lost at relapse is compared ($P=0.0148$), red dot, mean. E) The *RUNX1-RUNX1T1/ABL* qPCR ratios were comparable at diagnosis and relapse: shown is the change of *RUNX1-RUNX1T1/ABL* ratios for each patient at diagnosis and at relapse (n.s., $P=0.117$).

inferior prognosis.[22] Our analysis includes only 1 patient who relapsed following MR who had 2 signaling mutations at diagnosis (*FLT3*-TKD and *FLT3*-ITD) and thus cannot shed light on the role of clonal interference in this context.

The availability of paired samples at relapse allowed us to query the persistence and acquisition of mutations during clonal evolution of the initial AML clone. Interestingly when analyzing paired samples we observed a net loss of co-mutations with *RUNX1-RUNX1T1* across all patients: by a 63 gene panel analysis both the absolute number of mutations and the number of patients with co-mutations at relapse were significantly reduced. This is either due to subclonal architecture of AML with *RUNX1-RUNX1T* fusion and the selection of an ancestral subclone present at diagnosis or a branching evolution within a clone of the diagnostic sample. Single cell sequencing and the use of larger panels or whole exome/whole genome sequencing could further shed light on the nature of the clonal evolution.

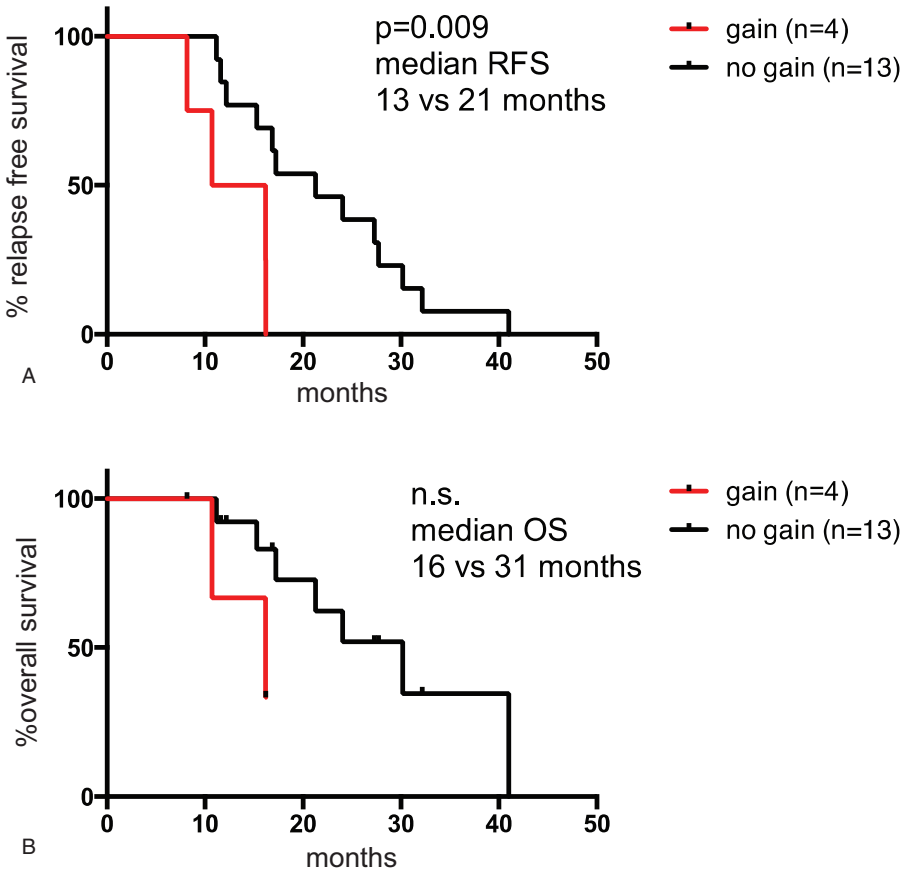


Figure 5. A gain of co-mutations at relapse is associated with reduced RFS. A) RFS of patients with no gained co-mutations (n=13) vs patients with gained co-mutations at relapse (n=4, $P=0.009$). B) Overall survival (OS) of patients with no gained co-mutations vs patients with gained co-mutations at relapse (n.s.).

It is important to note that in our analysis *RUNX1-RUNX1T1* is the key driver and stable over time. Therefore *RUNX1-RUNX1T1* fusion is the essential aberration for this leukemia and a prerequisite for successful MRD monitoring.[3,4] On the other hand *RUNX1-RUNX1T1* transcripts have been shown to persist in clinically healthy subjects for many years which indicates that *RUNX1-RUNX1T1* fusion is necessary but not sufficient to cause full AML.[24,25] What is more, preclinical models of core binding factor AML with *RUNX1-RUNX1T1* fusion established that the translocation causes enhanced replating efficiency of myeloid progenitors, but is not sufficient to induce leukemia.[26] These findings suggest a second hit scenario where the *RUNX1-RUNX1T1* fusion is the first hit that necessitates a second mutation to drive leukemia onset. In this light the loss of co-mutations in our analysis is counterintuitive, as a gain in driver mutations is generally believed to be associated with transformation and disease onset.[23] One explanation is that the true nature of required 2nd hit mutations in this AML entity has to be elucidated and that prototypic AML mutations detected by us and others[8–10] are only passenger mutations in this setting. Although signaling mutations are established 2nd hit driver mutations in AML[22,27] we observed a loss of *FLT3*, *KIT*, *KRAS* and *NRAS* mutations at relapse. In the same, line is an analysis of core binding factor AML at diagnosis and relapse that showed an increase of copy number variations at relapse and a gain of mutations in genes that are not commonly associated with leukemia.[28]

The VAF of mutations at diagnosis and relapse was not generally different. However, the VAF of mutations at diagnosis that were eventually lost was significantly lower than the VAF of mutations that persisted. This could be due to lower disease burden at relapse and mutations persisting at a lower level than our detection limit of 1%. By *RUNX1-RUNX1T1* ratio the disease burden was not significantly lower at relapse than at diagnosis and what is more we also observed mutations with a high allele frequency at diagnosis that were lost at relapse. In addition mutations that persisted did so with a comparable VAF at diagnosis and at relapse. Only 4/17 patients that had paired samples available for sequencing were evaluated by central morphology at relapse. We show that clonal loss is also observed in those patients with morphologic evidence of relapse. Taken together, this argues for the loss of an unfit clone at the advantage of a more aggressive clone that causes relapse of disease.

While the majority of patients had a loss of co-mutations at relapse, we observed a gain of mutations in 4 patients in 3 genes: *KIT*, *PHF6* and *TET2*. Gains in mutations were associated with reduced RFS and OS, however, this should be viewed with caution given the low number of patients. The gain of a mutation in *KIT*, *PHF6* or *TET2* could represent a true driver mutation that causes relapse in those patients.

Using a panel of 63 genes we show that the majority of co-mutations were lost at relapse. Therefore our data based on a highly selected subgroup of patients supports the notion that those mutations were not the key second hits driving relapse in

those patients. The fact that a higher number of mutations at diagnosis is associated with higher risk of relapse and that at relapse the majority of mutations are lost is counterintuitive. This could be explained by an increased number of mutations at diagnosis being a surrogate for mutational vulnerability of the present AML clone and the outgrowth of a more aggressive clone at expense of the initial leukemic clone at relapse.

Our analysis is somehow limited as we applied a targeted sequencing approach and investigated only 63 genes. We, therefore, miss deeper insights into the relevant second genetic hits in AML with *RUNX1-RUNX1T1* fusion. To completely decipher further mechanisms we propose a whole exome or whole genome sequencing strategy to identify the underlying genetic alterations that really drive this leukemia at diagnosis and at relapse. Knowledge about the true drivers might help tailor treatment or design elegant targeted treatment approaches in case of relapse.

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