Molecular characterization of acute myeloid leukemia patients who relapse more than 3 years after diagnosis: an exome sequencing study of 31 patients

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doi:10.3324/haematol.2019.227702

Supplemental Data

Methods

Routine diagnostics

Routine diagnostics (cytomorphology, cytogenetics and molecular assays including *NPM1* and *FLT3* mutation analyses) were performed as previously published.[1-4] Patients agreed with the use of laboratory data for research studies. The study followed the rules of the Helsinki Declaration.

Variant filtering

Data were processed with BaseSpace using the BWA Enrichment app with BWA for Alignment (against hg19) and GATK for variant calling with default parameters. Data were subsequently loaded into BaseSpace Variant Interpreter (Illumina) to filter and prioritize variants of interest. Only passed protein changing variants were considered with an ExAC population frequency of less than 1% for further analysis. To reduce the number of variant calls, we performed stringent filtering (Supplemental Figure 1). We excluded variants in genes known to be error-prone and certain gene families (*COL, FAM, GOL, KIR, KRT, PRB, PRS, ZNF, ADAM, OR* gene family members) where we also experienced a high number of potentially erroneous calls. In patients with remission samples, we excluded mutations with variant allele frequencies (VAFs) >10% in the matched remission sample from further analyses. Furthermore, we dismissed variants that were present in any remission samples (pooled germline control). In a separate step, we aimed to detect somatic mutations that persist at high VAFs (>10%) during permission. In order to avoid confounding by private germline variants, mutation calls in the remission sample were only included if they affected genes known to be frequently altered in AML and if they were previously described in the COSMIC database and there not classified as SNP. In patients without remission sample, we excluded variants also called in the pooled germline control. Moreover, dismissed SNVs if they were classified as 'benign' according to PolyPhen prediction tool.[5] Four patients received an allogeneic SCT prior to relapse. In order to distinguish relapse-specific mutations in transplanted patients from donor-cell

contamination, we excluded all variants called in the post-SCT remission sample from further analyses.

Results

Summary of patient characteristics

The cohort included 15 females and 16 males, aged 21 –75 years (median: 60 years) (Supplemental Table 1). Relapse occurred 3.0-8.1 years after diagnosis (median: 4.0 years) and complete remission was detected 0.1-5.0 years (median: 1.2 years) upon initial diagnosis. According to MRC criteria, patients were assigned to the following cytogenetic risk groups (cytogenetic data was not available for 2 patients): good risk, n=4 (14%, two patients with *RUNX1-RUNX1T1*, one patient with *CBFB-MYH11* and one patient with *PML-RARA*); intermediate risk, n=22 (76%) and adverse risk, n=3 (10%, two patients with complex karyotype, one patient with *KMT2A*-rearrangement). Furthermore, routine diagnostics identified *NPM1* mutations and *FLT3*-ITDs in 5/29 (17%) and 13/31 (42%) of tested patients, respectively. When comparing patients that relapse within the first year after diagnosis to our cohort of patients with relapse after >3 years, we observed a trend towards a lower frequency of high-risk genetic parameters in the latter group (adverse risk cytogenetics 10% vs 18%, p=0.44; *FLT3*-ITD, 17% vs 32%, p=0.14; *TP53, ASXL1* or *RUNX1* mutation 17% vs 31%, p=0.14) (Supplemental Table 2). However, this trend was not statistically significant.

Mutations occurring after chemotherapy

Transversions have been previously described to be enriched in AML patients who received chemotherapy. Overall, 142/524 (27%) of SNVs resulted from an A/T→C/G, C/G→A/T, C/G→G/C or A/T→T/A exchange (Supplemental Figure 3). We observed a significant increase in transversions when comparing mutations present in the diagnostic sample (84/386, 22%) compared to mutations acquired after chemotherapy (58/137, 42%), ($p<0.0001$), with the highest gain in A/T→T/A transversions (19% vs 33%, p=0.076). The transversion-rate among relapse specific mutations was comparable between patients who relapse within 3-4 years (15 patients; 28/70 of mutations, 40%) and 4-5 years (10 patients; 15/38 of mutations,

40%) but was increased in patients who relapse more than 5 years after diagnosis (6 patients, 15/29 mutations, 52%), however this was not statistically significant.

Case studies

Case 1: AML-15

The clonal evolution of molecular and cytogenetic alterations in AML patients with late relapses is best explained using specific cases. Patient AML-15 was diagnosed with AML M4 at 71 years of age. Routine diagnostics identified an *NPM1* mutation and a normal karyotype. The patient was negative for *FLT3*-ITD and received eight cycles of azacitidine. Relapse occurred 1241 days (3.4 years) after initial diagnosis. At this time point, cytogenetics identified an aberrant karyotype with a balanced translocation between the short arm of chromosome 1 and the long arm of chromosome 20 (46,XX,t(1;20)(p36;q11)). Exome sequencing identified a total of 25 mutations. Of these, six mutations were detected in the diagnostic and relapse sample while three mutations were present only at diagnosis and 15 mutations were acquired during disease progression (Supplemental Figure 4A). A remission sample was available and revealed persistence of an *IDH1* p.Arg132His mutation with a VAF of 63% at 62x coverage (VAFs at diagnosis and relapse: 42% and 55%, respectively). Interestingly, we observed an *SRSF2* mutation (c.284C>T, p.Pro95Leu) in this patient, which was neither present at diagnosis nor relapse but detected with a VAF of 31% (coverage: 155x) in the remission sample, indicating emergence of a clone with *SRFS2* mutation which is unrelated to the leukemic clone and repressed at relapse.

Case 2: AML-13

Patient AML-13 presented at an age of 39 years with an AML M4 and *KMT2A-MLLT3* rearrangement, routine molecular assays were negative for *NPM1* mutation and *FLT3*-ITD. The patient was initially treated with daunorubicin and cytarabine, resulting in complete cytogenetic remission and subsequently received a transplant from an HLA-matched donor. Relapse was evident 1246 days (3.4 years) after diagnosis. Donor cell leukemia was ruled out since the relapse sample was positive for the previously identified *KMT2A-MLLT3* rearrangement. Clonal evolution from diagnosis to relapse was identified by exome sequencing. A total of five mutations were

detected at both time points, including a *KRAS* p.Gly12Asp mutation (VAF at diagnosis: 17%, VAF at relapse: 43%). By contrast, an *NRAS* p.Gln61His mutation with a VAF of 28% at diagnosis was absent in the relapse sample (Supplemental Figure 4B). Aberrations gained during disease progression included a mutation in the transcription factor *SPI1* (p.Lys248Gln), which is predicted to affect the DNA-binding domain, as well as a truncating mutation in *G2E3*, a ubiquitin ligase involved in apoptotic processes which was descripted to be affected in *RUNX1-RUNX1T1* positive AML.

Case 3: AML-9

Patient AML-9 was diagnosed with AML at 37 years of age, harboring a *NPM1* mutation and a normal karyotype. The patient was treated with intensive chemotherapy but relapsed after 1402 days (3.8 years). Exome sequencing identified three mutations shared between diagnostic and relapse sample. A total of nine mutations were lost at relapse, including a *NRAS* p.Gln61Lys mutation (VAF at diagnosis: 44% with 55x coverage) and five mutations, including a truncating mutation in transcription factor *ETV6*, were gained (Supplemental Figure 4C). The patient received an allogeneic transplant and was four years later still in remission.

Case 4: AML-32

Patient AML-32 presented with AML M2 at the age of 57 years. An *NPM1* type A mutation was detected and cytogenetics identified a deletion in the long arm of chromosome 9. The patient was treated with idarubicin, cytarabine, etoposide + all-trans retinoic acid (ATRA) and went into complete morphological as well as molecular remission (*NPM1* mutation not detectable at remission). Treatment was continued with three cycles of cytarabine /ATRA. The patient relapsed after 2649 days (7.3 years). Exome sequencing identified a total of 19 mutations at diagnosis and two mutations at relapse (Supplemental Figure 4D). AML-32 was positive for the *NPM1* mutation both at diagnosis and relapse, but lacked other shared gene mutations between the two time points. Similar, cytogenetic analyses demonstrated two independent clones between both disease time points (46,XX,del(9)(q22q34) at diagnosis and 46,XX,t(2;6)(p24:q11) at relapse). It is unclear whether this patient presented with two genetically unrelated AML clones that both happened to carry an *NPM1* mutation (which is one of the most frequent genetic aberration in AML

patients, we observed the identical mutation at diagnosis and at relapse) or if the *NPM1* mutation was a very early event in this patient and was the origin for the relapse clone which otherwise acquired a new set of further genetic lesions.

We here presented several case studies to depict the clonal evolution in AML patients with late relapses. In one particular case, we detected an *SRSF2* mutation that was exclusively present the remission sample. Similarly, another study reported cases with expansion of non-leukemic clones after induction chemotherapy.[6] In our patient, the non-leukemic clone was repressed by the emerging relapse clone, indicating inferior fitness. In another case, we observed two (cyto)genetically distinct clones between diagnosis and relapse. Both clones, however, were positive for the same *NPM1* mutation. It remains uncertain whether these are truly two related clones that share a common ancestral clone with *NPM1* mutation, or if we observed two distinct diseases (de novo AML and therapy-associated AML) that happened to both be positive for an *NPM1* mutation, one of the most frequent aberrations in AML.

References

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Supplemental Tables

Supplemental Table 1: Patient characteristics

Age at diagnosis of AML (years)	
Median	60
Range	21-75
Sex	
Female	15 (48%)
Male	16 (52%)
Cytogenetic risk group at diagnosis according to MRC criteria	
Not available	2
Good	4 (14%)
Intermediate	22 (76%)
Adverse	3 (10%)

MRC: Medical Research Council

Supplemental Table 2: Frequency of high-risk genetic features at diagnosis of AML among patients who relapse within 1 year or >3 years after diagnosis.

Characteristics	Relapse > 3 years (n=31)	Relapse < 1 year (n=371)	p-Value
Adverse risk cytogenetics	3/29 (10%)	66/371 (18%)	0.445
FLT3-ITD	5/29 (17%)	115/362 (32%)	0.142
Adverse risk gene mutations (*TP53, ASXL1, RUNX1*)	5/29 (17%)	94/301 (31%)	0.140

ITD= internal tandem duplication

Supplemental Table 3: Number of called variant per patient and time point

Patient	Remission sample available	Total mutations in exome	Time point of mutation calling			
			Diagnosis and relapse	Diagnosis only	Relapse only	Persistent in remission
AML-01	yes	11	4	4	3	0
AML-02	yes	18	11	2	5	0
AML-03	no	21	15	2	4	0
AML-04	no	17	9	1	7	0
AML-05	yes	8	3	1	4	0
AML-06	no	23	20	1	2	0
AML-07	yes	27	12	2	12	1
AML-09	yes	17	1	9	5	2
AML-10	yes	9	1	6	2	0
AML-11	no	23	17	3	3	0
AML-12	yes	4	1	3	0	0
AML-13	yes	13	5	2	6	0
AML-15	yes	25	5	3	15	2
AML-16	no	30	20	4	6	0
AML-17	no	20	15	4	1	0
AML-18	yes	16	0	6	8	2
AML-19	yes	19	8	2	9	0
AML-20	no	17	12	2	3	0
AML-21	yes	15	3	4	7	1
AML-23	no	18	16	1	1	0
AML-25	no	16	13	0	3	0
AML-26	yes	24	8	4	12	0
AML-27	no	26	23	2	1	0
AML-28	no	12	10	1	1	0
AML-29	yes	27	15	2	10	0
AML-30	no	21	20	0	1	0
AML-31	yes	34	14	5	15	0
AML-32	yes	22	0	19	3	0
AML-33	yes	25	7	6	11	1
AML-34	yes	24	8	7	9	0
AML-35	yes	8	1	6	1	0
Sum		590	297	114	170	9
Min		4	0	0	0	0
Max		34	23	19	15	2
Median		19	9	3	4	0

Supplemental Table 4: Cytogenetics at time point of diagnosis and relapse

Patient	Diagnosis	Relapse
AML-01	46,XY[20]	46,XY[20]
AML-02	46,XX[20]	46,XX[20]
AML-03	45,XY,del(5)(q15q34),-7,t(11;21)(q13;q11),del(12)(p12p13)[15]	46,XY,t(11;21)(q13;q11)[6]/46,XY,del(5)(q15q34),t(11;21)(q13;q11)[3]/45,XY,del(5)(q15q34),-7,t(11;21)(q13;q11)[5]/45,XY,del(5)(q15q34),-7,t(11;21)(q13;q11),del(12)(p12p13)[4]
AML-04	47,XX,+11[20]	NA
AML-05	46,XY[20]	46,X,del(Y)(q11)[10]
AML-06	46,XY[20]	46,XY[20]
AML-07	NA	46,XY[20]
AML-09	46,XY[20]	46,XY,del(11)(p12p14)[16]
AML-10	46,XY,t(15;17)(q24;q21)[2]/46,XY,der(15)t(15;17)(q24;q21),ider(17)(q10)t(15;17)(q24;q21)[9]	48,XY,der(15)t(15;17)(q24;q21),ider(17)(q10)t(15;17)(q24;q21),+ider(17)(q10)t(15;17)(q24;q21),+21[15]
AML-11	46,XX[20]	46,XX[20]
AML-12	46,XY,t(6;21;8)(q15;q22;q22)[20]	46,XY,t(6;21;8)(q15;q22;q22)[11]
AML-13	47,XY,+8,t(9;11)(p22;q23)[19]	47,XY,+8,t(9;11)(p22;q23)[3]
AML-15	46,XX[20]	46,XX,t(1;20)(p36;q11)[14]
AML-16	47,XX,+8[11]/48,XX,+8,+13[7]	48,XX,+8,+13[2]/91,XXXX,der(5)t(5;6)(q14;q??),-6[2]
AML-17	47,XY,+8[5]	45,X,-Y[7]/47,XY,+8[4]
AML-18	46,XX[20]	46,XX[20]
AML-19	46,XX[20]	46,XX[20]
AML-20	46,XY,t(8;21)(q22;q22)[3]/46,XY,t(8;21)(q22;q22),t(9;11)(q34;q13)[9]/45,X,-Y,t(8;21)(q22;q22),t(9;11)(q34;q13)[7]	46,XY,t(8;21)(q22;q22),t(9;11)(q34;q13)[20]
AML-21	46,XX[20]	47,XX,+21[19]
AML-23	46,XY[20]	46,XY[20]
AML-25	47,XX,+13[2]	47,XX,+13[8]
AML-26	NA	46,XX[20]

AML-27	46,XX[20]	47,XX,+8[9]
AML-28	46,XY,del(16)(q13q24)[9]	46,XY,del(16)(q13q24)[6]/46,XY,der(10)t(1;10)(q21;p15), del(16)(q13q24)[12]/46,XY,der(7)t(1;7)(q21;p22),del(16)(q13q24)[3]
AML-29	46,XX[20]	46,XX,t(1;21)(q23;q22)[17]
AML-30	47,XY,der(3)t(3;13)(p14;q21),dic(5;7)(q14;q22), der(6)t(5;6)(?;p12)t(3;5)(?;?),+11,der(13)t(6;13)(?;q13), der(13)t(11;13)(q23;q31),der(20)t(1;20)(?;q11),+22[15]/ 47,XY,der(3)t(3;13)(p14;q21)t(3;9)(q27;q32), dic(5;7)(q14;q22),der(6)t(5;6)(?;p12)t(3;5)(?;?), der(9)t(3;9)(q27;q32),+11,der(13)t(6;13)(?;q13), der(13)t(11;13)(q23;q31),der(20)t(1;20)(?;q11),+22[2]	52,XY,+Y,der(3)t(3;13)(p14;q21),+5,dic(5;7)(q14;q22), der(6)t(5;6)(q?34;p12)t(3;5)(p14;q?35),+8,+11, +der(13)t(6;13)(p22;q13),der(13)t(11;13)(q23;q31)x2,+14, der(20)t(1;20)(?p32;q11),+22[9]
AML-31	46,XX[20]	46,XX[20]
AML-32	46,XX,del(9)(q22q34)[11]	46,XX,t(2;6)(p24:q11)[8]
AML-33	46,XY[20]	47,XY,+8[17]
AML-34	46,XX,t(1;3)(p36;q21)[19]	46,XX,t(1;3)(p36;q21)[19]
AML-35	46,XY,inv(16)(p13q22)[16]	NA

NA: not analysed

Figure Legends

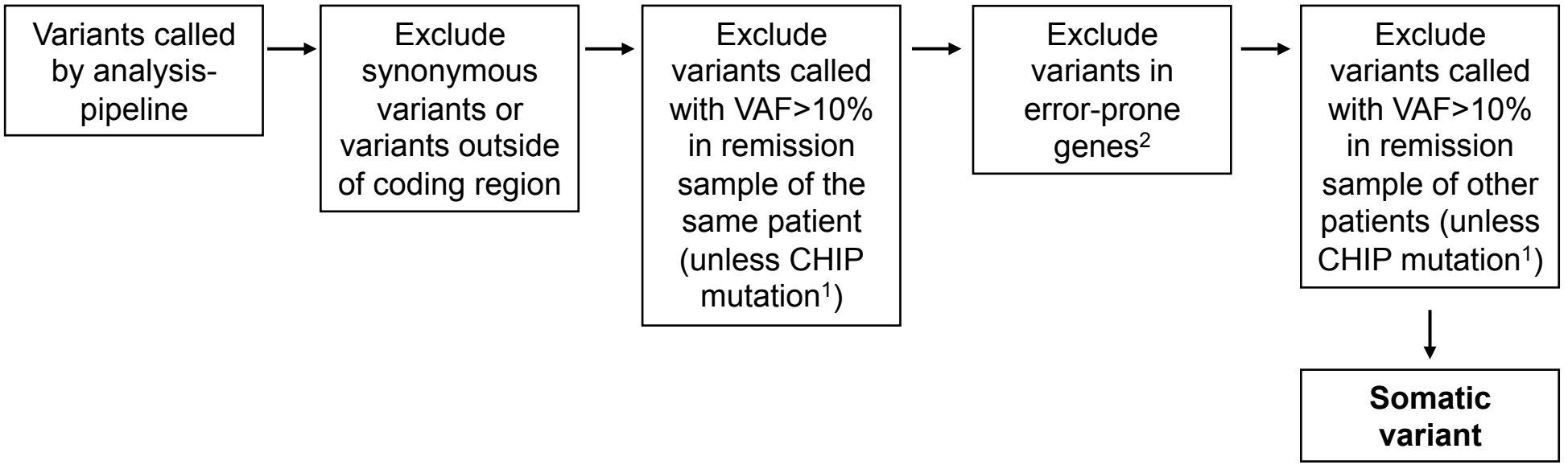
Supplemental Figure 1: Overview of variant filtering. (A) Schematic description of variant filtering steps performed in samples with matched remission as control. (B) Schematic description of variant filtering steps performed in samples without matched remission as control.

Supplemental Figure 2: Mutation frequency according to function of the affected molecule. PANTHER gene list analysis was performed to categorize the identified gene mutations. Blue bar: all detected mutations. Light grey bar: mutations detected at diagnosis and absent in the matched relapse sample. Dark grey bar: mutations detected at relapse and absent in the matched diagnostic sample.

Supplemental Figure 3: Mutation metrics. A. Distribution of SNVs and INDELs among all 590 identified mutations. B. Time point of mutation detection. Diagnostic and matched relapse samples of each patient were compared and mutations classified according to which time point they were present. C. Frequency of transversions among all 524 identified SNVs. The frequency was compared between mutations that were present at diagnosis and mutations aquired at relapse.

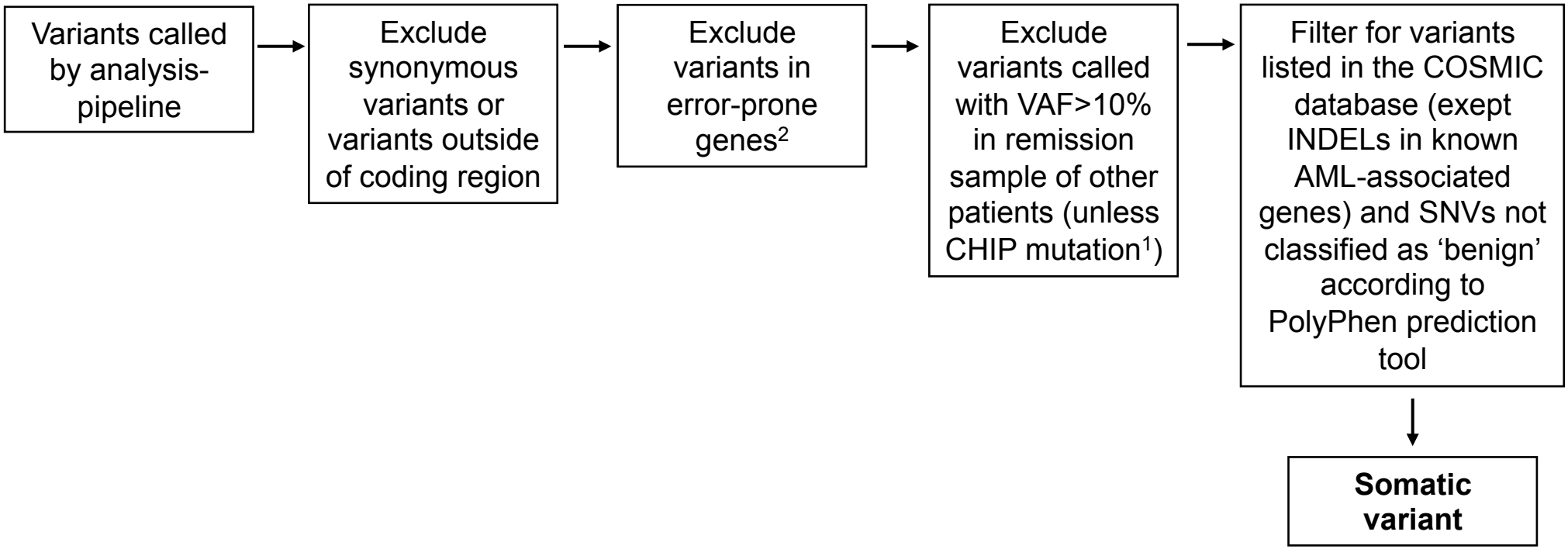
Supplemental Figure 4: Clonal evolution from diagnosis to relapse of four different patients. Red lines: mutations present at diagnosis and relapse. Light grey lines: mutations lost at relapse. Dark grey lines: mutations gained at relapse. The karyotype observed at diagnosis and relapse is also depicted. A. Patient AML-15 with independent *SRSF2* mutated clone (blue line) present only in the remission sample. B. Patient AML-13 with relapse after transplantation. C. Patient AML-9 with lost mutation in *NRAS* (signaling pathway) and gained mutation in *ETV6* (transcription factor). D. For patient AML-32, molecular analyses demonstrated an *NPM1* mutation at both time points (not indicated as mutation load was not determined by routine diagnostics), otherwise this patient showed no additional shared mutations between diagnosis and relapse.

Supplemental Figure 1A



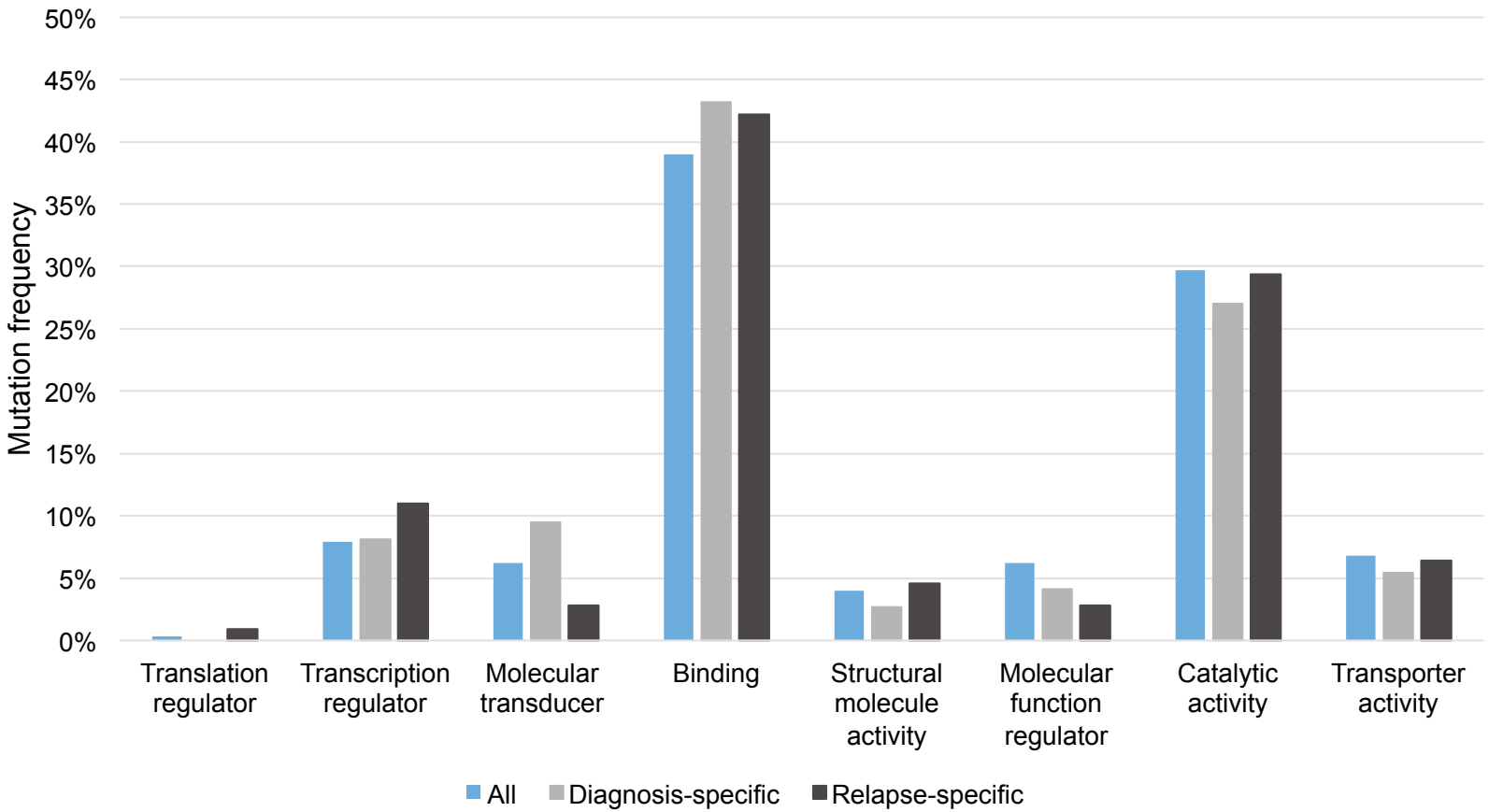
[1] CHIP: clonal hematopoiesis of indeterminate potential, mutations e.g. in *DNMT3A, IDH, ASXL1, TET2*, etc.
[2] according to Fuentes Fajardo et al (2012)

Supplemental Figure 1B

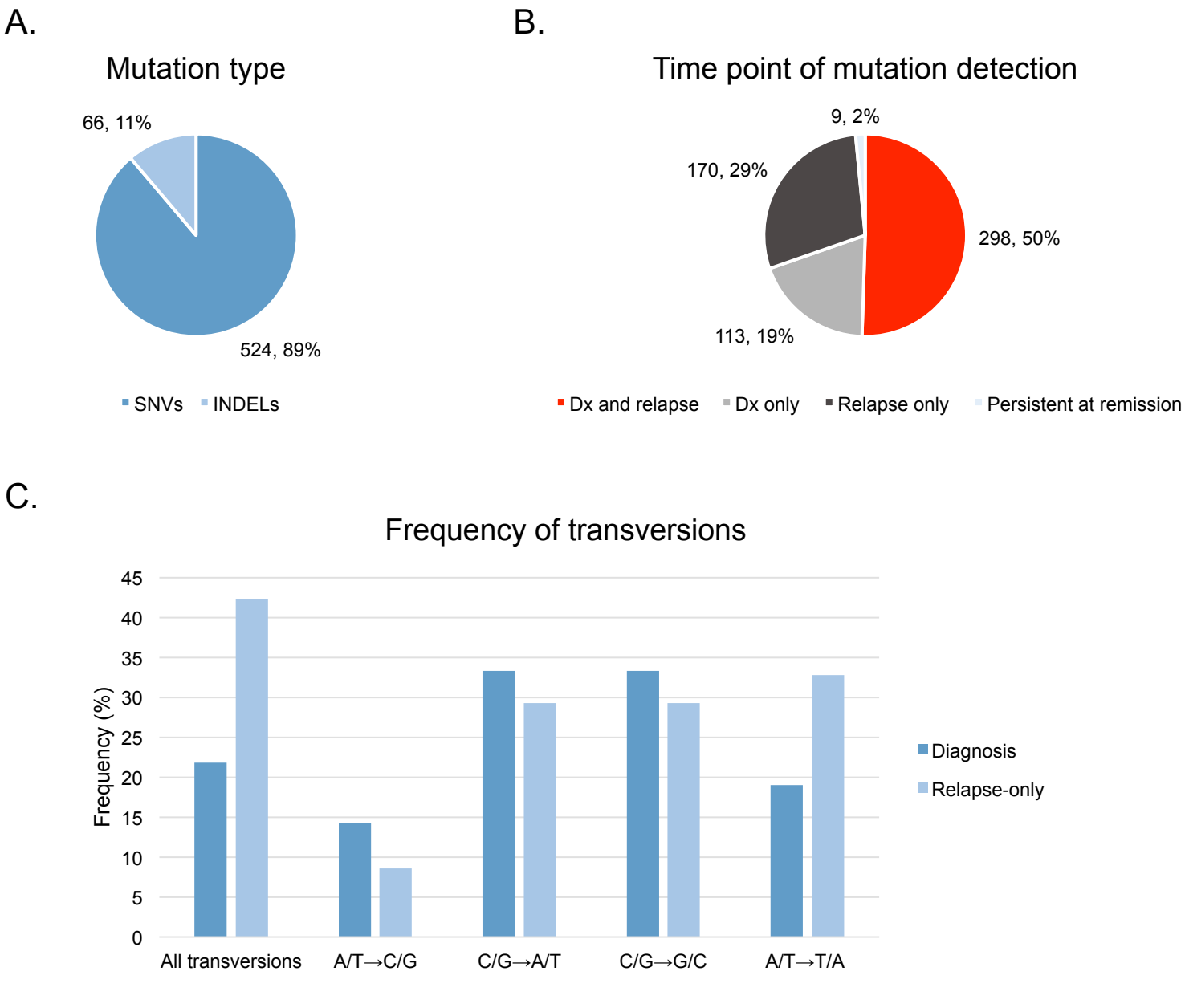


[1] CHIP: clonal hematopoiesis of indeterminate potential, mutations e.g. in *DNMT3A, IDH, ASXL1, TET2*, etc.
[2] according to Fuentes Fajardo et al (2012)

Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4