Somatic mutations of activated signaling genes, transcription factors, or tumor suppressors are a precondition for leukemic transformation from myelodysplastic syndromes: a sequencing analysis of 64 paired samples.

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This study was supported by the National Natural Science Foundation of China (grant nos. 81770120 and 81770122).

Abstract

The transformation biology of secondary AML from MDS is still not fully understood. Here, we performed a large cohort of paired sequences including target, whole-exome and single cell sequencing to search AML transformation- related mutations (TRM). The results showed that fifty-five out of the 64 (85.9%) patients presented presumptive TRM involving activated signaling, transcription factors, or tumor suppressors. Most of TRM (63.6%, 35 cases) emerged at the leukemia transformation point. All five of the remaining nine patients analyzed by paired whole exome sequencing showed TRM which are not included in the reference targets. Single-cell sequencing indicated that the activated cell signaling route was related to TRM which take place prior to phenotypic development. Of note, defined TRM was limited to a small set of genes (less than ten, in the order: NRAS/KRAS, CEBPA, TP53, FLT3, RUNX1, CBL, PTPN11 and WT1, accounted for 91.0% of the mutations). In conclusion, somatic mutations involving in activated signaling, transcription factors, or tumor suppressors appeared to be a precondition for AML transformation from myelodysplastic syndromes. The TRM may be considered as new therapy targets.

In most cases, *de novo* acute myeloid leukemia (AML) shows rapid onset without an obvious pre-AML period [1]. Patients have been reported to show genetic abnormalities, such as PML/RARa in the M3 subset [2]; ETO fusion gene in the M2b subset [3]; and CBFβ-MYH11 in M4 EO [4]. In addition, somatic mutations involving CEBPa, FLT3, NPM1, and c-Kit have been used to assess prognosis [5]. Based on these tumor-derived biological characteristics, target-specific and immunological methods have been developed to treat AML [6]. Unlike *de novo* AML, secondary AML (sAML), transformed from myelodysplastic syndromes (MDS), shows unique genetic features. First, there is often an obvious pre-AML stage, where somatic gene mutations result in initial events (clonal hematopoiesis) and/or driver events (development of MDS phenotypes), largely involving epigenetic regulation, RNA splicing, fewer transcription, and others [7,8]. Second, despite preliminary findings on the roles of late-stage gene mutations involving various signaling pathways or transcription during sAML development [9,10], including our primary consideration on sAML-related mutations [11], the transformation biology of sAML is still not fully understood. There are many unanswered questions, as: Could early or late somatic mutations solely involved in epigenetic regulation or RNA splicing also induce sAML? In other word, if it is necessary for sAML transformation by occurrence of some more aggressive gene mutations? Which mutations are the key factors that induce the transformation process? When do the transformation-related mutations emerge? Are they pre-existing at MDS diagnosis, or do they emerge when the transformation starts? Because of the lack of conclusive finding on sAML transformation, we tentatively named those suspected last events as sAML transformation-related mutations (TRM). Hypothesizing that mutations involving signaling pathways, myeloid transcription factors, or tumor suppressors at MDS diagnosis and AML transformation are both a precondition for MDS to progress to sAML, we proposed that analyzing a

large cohort of paired samples would help answering to these questions. Examination of over 1,000 MDS patients over 10 years, we identified 64 patients who underwent AML transformation. Paired samples were acquired at MDS diagnosis and immediately after AML transformation. Sequencing for 39 target genes in all samples was followed by exome sequencing in the few patients whose target sequencing did not identify novel mutations. An additional sample pair was subjected to single-cell RNA transcription sequencing. Using these techniques, we obtained data that informs the biology of sAML transformation and suggests novel strategies to prevent leukemic transformation of MDS.

Patients and Methods

Sample collection

From January 2004 until October 2019, a total of 1,427 patients were diagnosed with MDS in our department. Over 90% were followed up by calls or with face-to-face consultations, and over 800 patients consented to provide samples for next generation sequencing. DNA was extracted from bone marrow (BM) samples obtained from patients before 2009 and retrospectively used for target sequencing or whole exome sequencing. For patients whose BM samples were acquired in or after 2009, DNA was extracted from the samples and immediately used for targeted sequencing or whole exome sequencing. For single-cell RNA transcription sequencing, fresh BM was used to isolate mononuclear cells within six hours of BM aspiration. Targeted sequencing was performed using paired samples (at the diagnosis and transformation points) to detect transformation-related gene mutations (TRM). Whole exome sequencing was performed when conclusive results for last mutation events were not obtained via targeted sequencing and when adequate residual DNA extracts were available. Single-cell RNA transcription sequencing was used to examine the underlying transformation dynamics. Diagnoses for MDS and sAML were established in strict accordance with the WHO criteria, and the CMML subset was diagnosed according to the FAB classification [12,13].

Genomic DNA preparation, target enrichment, and sequencing

Genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer’s protocol. Genomic DNA was sheared using the Covaris® system (Covaris, USA), and the DNA sample was prepared using the Truseq DNA Sample preparation Kit (Illumina, USA) according to the manufacturer's protocol.

For probe design, both coding and regulatory regions of target genes were

included in the custom panel. The regulatory regions comprised promoter regions (defined as 2 kb upstream of the transcription start site), 5′ un-translated region (5′-UTR), and intron-exon boundaries (50 bp). Custom capture oligos were designed using SureDesign website of Agilent Technologies (Agilent, USA). Hybridization reactions were carried out on ABI 2720 Thermal Cycler (Life Technologies, USA) with the following hybridization conditions. Hybridization mixture was incubated for 16 or 24 h at 65°C with a heated lid at 105°C. After the hybridization reactions, the hybridization mixture was captured and washed with magnetic beads (Invitrogen, USA) and SureSelect target enrichment kit (Agilent, USA). The captured product was enriched with the following cycling conditions, 98ºC for 30s, 10 cycles of 98ºC for 10 s, 60ºC for 30 s, 72ºC for 30 s, and 72ºC for 5 min. Library quality was assessed using an Agilent 2100 Bioanalyzer (Agilent, USA), and multiplexed sequencing was performed on HiSeq 2500 sequencers with 2×150 paired-end modules (Illumina, USA). Average sequencing depth was 800×. Following 39 sequenced target genes were included: *ANKRD11, ASXL1, BCOR, CALR, CBL, CEBPA, DHX9, DNMT3A, ETV6, EZH2, FLT3, GATA2, IDH1, IDH2, ITIH3, JAK2, KIF20B, KIT, KRAS, MPL, NF1, NPM1, NRAS, PHF6, PTPN11, PTPRD, ROBO1, ROBO2, RUNX1, SETBP1, SF3B1, SRSF2, STAG2, TET2, TP53, U2AF1, UPF3A, WT1*, and *ZRSR2*.

Whole-exome sequencing

The gDNA library was prepared using a TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA, US) in accordance with the manufacturer's protocol. In-solution exome enrichment was performed using a TruSeq Exome Enrichment kit (Illumina) according to the manufacturer's instructions. The enriched DNA samples were sequenced via 2×100 paired-end sequencing using a Hiseq2000 Sequencing System (Illumina). Illumina Sequencing Control v2.8, Illumina Off-Line Basecaller v1.8, and Illumina Consensus

Assessment of Sequence and Variation v1.8 software were used to produce 100-base pair (bp) sequence reads.

Sequencing data processing, variant calling, and annotation

Before variant calling, the raw sequence reads were mapped to the reference genome (hg19), duplicate reads were marked and removed to mitigate biases introduced by amplification, and base quality scores were recalibrated using the Genome Analysis Toolkit (GATK). The Ensembl VEP and vcf2maf tools were applied to generate a MAF format for somatic mutation annotation, and ANNOVAR tool was used to annotate frequency information of variations in the population database. The variants were identified as low-frequency functional mutation if they had < 0.1 frequency in ExAC03 database, < 0.01 frequency in 1,000 genome database, and < 0.05 frequency in GeneskyDB database. According to the results, the variant was then extracted as one of the following functional annotations: "Frame_Shift_Del/ins", "In_Frame_ Del/Ins", "Missense_Mutation", "Nonsense_Mutation", "Nonstop_Mutation", "Splice_Site" or "Translation_Start_Site".

Single-cell RNA sequencing and bioinformatic data analysis

Bone marrow mononuclear cells(BMMC) concentrations were measured by hemocytometer and adjusted to 700-2,000 cells/μL. Single-cell RNA-seq libraries were generated using 10x Genomics Single Cell 3' reagent v2 according to the manufacturer's instructions. Twelve and 14 cycles were used for complementary DNA and index PCR amplifications, respectively. Amplified cDNA and library quality was assessed using Agilent 2100 Bioanalyzer (Agilent, USA). Libraries were pooled and sequenced on HiSeqX Ten platform (Illumina) with 2×150 paired-end modules generating at least 50K mean reads per cell. Raw sequencing data were processed using Cell Ranger version 3.1.0 (10x Genomics) with default parameters. Quality control metrics

were used to remove cells with mitochondrial gene percentage more than 20% or cells with fewer than 200 genes detected. Variably expressed gene selection, dimensionality reduction, and clustering were performed using the Seurat package version 3.1.3. Principal component analysis was performed on significantly variable genes and the first 40 principal components were used for UMAP dimension reduction. Cell type of each cluster was identified using singleR version 1.0.1. For paired samples, differentially expressed genes (fold change > 2 and Wilcox test P value < 0.05) were identified in each of seven cell types independently, of which the mutation genes PTPN11 and NRAS were used for GO and KEGG enrichment analysis.

Definition of the presumed transformation-related mutation

When we analyzed the paired data, if pre-existing genes at MDS diagnosis or newly-emerging genes at sAML transformation met following conditions, we considered they are sAML transformation related mutations: 1. they must be involved in at least one of the three function pathways namely, active signaling, myeloid transcription or tumor suppression. 2. They emerged after sAML transformation (better weight) or pre-existed at MDS diagnosis (poorer weight). Newly emerged mutations were preferentially considered as the presumed transformation-related mutations. 3. When ≥ 2 suspicious mutations co-existed to be defined as transformation-related mutations, the biologically more aggressive one (active signaling > myeloid transcription > tumor suppressor); or with lower VAF (Various allele frequency) among newly emerged mutations (meaning latest emergence) were defined as transformation-related mutations (TRM).

Statistics analysis

Statistical analyses were conducted using SPSS software version 18.0. Kaplan-Meier analysis was used to evaluate the time to survival and time to

progression. All P-values were based on 2-sided tests and P-values less than 0.05 were considered statistically significant.

Results

Target sequencing

Paired samples acquired from 64 MDS patients before and after transformation to sAML were analyzed using target sequencing. Identified somatic mutations are presented in Table 1.

1. Transformation-related mutations (TRM) were identified in 55 of 64 patients (85.9%), according to the steps described in METHODS.

2. Among these 55, TRM were detected only at AML transformation in 35 patients (representing 63.6% of these cases). In the 20 remaining cases, these mutations were detected at the point of diagnosis (Table 1). Patients with TRM in only their sAML sample trended toward a longer time to transformation (mean 13.4 months vs 9.6 months for the first; *P*=0.106) and no difference in lower risk (<RAEB2/CMML2) cases at diagnosis (80.0% vs 75.0% for the second; *P*=0.431).

3. From Table 1, 2 and Figure 1, the following defined TRMs were present in descending order of frequency. First were active signaling mutations:*KRAS*/*NRAS* in 11 patients (two also had *CBL* or *PTPN11* as TRM); *FLT3* in six patients (one also had CBL); *CBL* in six patients (three had *NRAS*; *FLT3* and *CEBPA* respectively); and *PTPN11* in four (one had *NRAS*); the remaining two had *PTPRD* and *GATA2*, respectively. The next set of mutations comprised myeloid transcription factors: *CEBPA* in 10 patients (one had *CBL*); *RUNX1* in five patients, and *ETV6* in two patients. The next set of mutations comprised tumor suppressor genes: *TP53* mutations in nine patients; *WT1* mutations in three patients, and *NPM1* in one patient. *KIT* mutations were not detected in this group of patients. The TRM events seemed to be highly enriched in about 10 genes. Figure 1 shows the pattern of these mutation events, demonstrating that the involvement style of *FLT3* was not the same as that of FLT3-ITD, which is more often observed in *de novo* AML.

4. In 6 of these 55 paired samples with candidate transformation events, we sequenced an additional sample between MDS diagnosis and AML transformation (Figure 2). Of these, 3 samples (UPN1243, 3288, and 4390) showed evidence of the TRM emerging before phenotype change, i.e., the TRM event emerged while the disease was still in the MDS stage, with sAML transformation occurring soon thereafter.

5. Analysis of changing mutation profiles and VAF, as well as the logical relations between them, revealed differing patterns of evolution from MDS to sAML. Of the 55 patients with candidate mutation events, most (25 cases) demonstrated AML-transformation-related progression by linear evolution from the founding clone (Figure 3a). Five cases showed linear evolution from subclones (Figure 3b). Only four cases demonstrated sweeping clonal evolution (Figure 3c). The evolution pattern of the remaining 21 patients either could not be defined by changes in mutations or VAF (4 cases), or harbored no candidate alterations before or after progression to AML.

6. Rivalry clonal evolution patterns were observed. Some original TRM were replaced or outgrown by other mutations at sAML transformation, such as in patients 2 (UPN3430), 5 (UPN809); 9 (UPN4603), 13 (UPN2570), and 54 (UPN3567) (Table 1)

7. Although differences existed among TRM, TET2/RUNX1/ASXL1 /DNMT3A /EZH2/U2AF1 mutations were the most common initial/driver mutations for these AML-transformed patients (Table2). However, TET2/ASXL1/BCOR /ROBO1 mutations were most commonly accompanied by those transformation-related events when transformation occurred (Table2).

Whole exome sequencing

Samples from five of the nine patients whose target sequencing showed no TRM but for whom sufficient DNA extract was still available were further subjected to whole exome sequencing (WES) (patients starred in Table 1).

Figure 4 presents the results of WES from these cases. In addition to MDS-related gene mutations, each gained at least one TRM (involving activated signaling, transcription factor, or tumor suppressor genes) at the AML transformation point. Specifically, UPN1601 and UPN 3053 acquired NOTCH1 and PAK5 mutations, respectively (both involved in signaling transduction), during disease progression. NOTCH1 mutations are reported to occur frequently in acute leukemia [14], whereas PAK5 contributes to the proliferation of tumor cells as serine/threonine protein kinase [15]. UPN1702 and UPN3831 acquired MLLT10 and NCOR2 mutations, respectively (both involved in transcription regulation), at the AML stage. MLLT10 is a histone lysine methyltransferase, which participates in AML pathogenesis via the formation of fusion gene MLLT10-MLL [16]. NCOR2 regulates gene transcription as a part of a histone deacetylases complex, and its dysfunction leads to functional abnormality in hematopoietic stem cells [17]. UPN3155 acquired a STK11 mutation (a tumor suppressor) after disease progression. It has been reported that STK11 regulates cell polarity and functions as a tumor suppressor [18].

Single-cell RNA sequencing

As mentioned above, abnormal cell signaling induced by gene mutation, such as RAS genes or *PTPN11* may be critical for the transformation of MDS into AML. However, it is still unclear whether a RAS mutation is a requirement to activate RAS signaling pathways. To explore this question, we used single-cell RNA transcription sequencing to study the association between RAS mutation and RAS signaling in UPN4674 and UPN4763 (before and after disease progression). Two s (*NRAS* and *PTPN11* mutations) occurring during the AML stage are core genes in RAS signaling. As shown in Figure 5a and 5b, this patient presented several abnormal cell types (orange, UPN4674; blue, UPN4763 in Figure 5a). Gene classification analysis showed that

aberrant granular-mononuclear progenitors (GMP), common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP) and monocytes (Mono) were present during the AML stage (Figure 5b). We focused on differences in the GMP population were RAS signaling. Integrated analysis based on single-cell sequencing indicated that several RAS signaling related-genes are expressed at high levels in GMP after disease progression (Figure 5c). These genes have been reported to participate in the activation of RAS signaling [19]. Similarly, RAS signaling related-genes such as *FLT3*, *INSR*, and *CDC42* are also expressed at high levels in MEP, CMP, and Mono groups after disease progression (Figure 5d). These genes are closely associated with cell proliferation. Interestingly, apoptosis-related gene *BAD* is down-regulated in GMP, MEP, CMP, and Mono groups after disease progression. These data suggest that transformation-related mutations gene mutations induce the redistribution of clonal cells (leading to an increased number of morphological blasts and monocytes) via the activation of cell signaling, and further leads to AML transformation.

Discussion

The pathogenesis of sAML differs from that of *de novo* AML in several respects including clinical progression and prognosis [20]. Despite poor response to contemporary therapies, including stem-cell transplantion, MDS-derived AML exhibits a relatively long pre-transformation duration, and occurs only in about one third of all MDS [7]. If this transformation requires triggering factors (defined as TRM), and these factors are robust biomarkers of progression, it may be possible to develop counter mechanisms to prevent transformation.

In this study, we sequenced 64 paired bone marrow DNA samples, first at MDS diagnosis and then immediately after AML transformation. Fifty-five out of the 64 cases (85.9%) were positive for TRM, i.e., events involving active signaling, transcription factors, and tumor suppressors. Sequencing of 39 target genes could not detect any TRM in 9 out of the 64 cases. Of these, paired whole exome sequencing was performed for 5 cases for which sufficient DNA could be extracted from BM samples. Each of these patients carried at least one TRM outside of the 39 target genes, involving active signaling, myeloid transcription, or tumor suppressor genes (Figure 4). No patient with MDS who developed sAML did so only by acquiring mutations involved in epigenetic modulation, RNA splicing, or cohesins.. This finding suggests that the existence or emergence of certain last event-like mutations may be considered a prerequisite for MDS to transform into sAML, rather than just occasional or accompanying events [9,10].

Among the 60 patients with detected TRM, in 40 patients (66.7%; including 35 by targeted sequencing and 5 by WES) the TRM appeared to emerge at the point of AML transformation, and the remaining 20 patients harbored putative last mutations at the time of MDS diagnosis. Then it is necessary to tell which the original is? The existence/emerge of TRM? Or the AML transformation itself? We considered that the pre-existence or emergence of

TRM should be the reason for AML transformation. First, 20/60 (33.3%) cases harbored TRM at MDS diagnosis; The duration of transformation was moderately shorter in these cases than in those where the TRM were only identified at the transformation point (mean 9.8 vs. 13.4 months). Second, due to the relatively longer corresponding intermittence between time points at diagnosis and AML transformation for paired sequencing, some important information may have been missed. When additional time points were added, supportive data were obtained from some patients (UPN1243, 3288, and 4390 in Figure 2). When TRM emerged in these patients, their disease was still at the low-risk or pre-AML stage, but transformed to AML quickly. Finally, for one patient (UPN 4603), immediately after the occurrence of the AML phenotype, accompanied by NRAS and PTPN11 mutations, single-cell RNA sequencing showed both active RNA transcription and activation of the RAS/PTPN11 signaling pathway [21,22]. Given that the gene mutations occurred prior to RNA/protein transcription/translation, pathway activation, and ultimately, phenotype alteration. These results support our findings that TRM and activated RAS signaling can precede phenotypic transformation to AML.

Most of the TRM clones showed linear evolution from the founding clones (25 cases, compared to 5 cases with linear evolution in a pre-existing subclone; and 4 cases by clone sweeping) (Figure 3). This is somewhat different from published data [23,24], may because of that a part of patients did not presented clonal progressing process, such as those 20 cases whose TRM pre-existed at MDS diagnosis. In addition, we detected common pre-existing mutations involving *TET2*/*RUNX1*/*ASXL1*/*DNMT3A* (Table 2), indicating that these mutations represent precursor mutations for these AML cases. As for *TET2*/*ASXL1*/*BCOR*/ *ROBO1* mutations, they emerged as common partners of TRM (Table 2), possibly playing some roles in the transformation process. Some mutated genes such as RUNX1 (highly

prevalent among patients with chromosome 7 abnormalities) and BCOR (common in chromosome normal cases) [25] could be responsible either for the MDS phenotype or driving AML progression. These dynamic genetic features contribute to the complexity and heterogeneity of this disease and may become a key to deciphering the rule for sAML occurrence.

Of note, the majority of detected TRM were from a cluster of 10 related genes. *KIT* mutation was never detected in this group of patients, and NPM1 was only detected in one case. The latter could be attributed to the favorable responses of the MDS patients who harbor NPM1 mutations towards HMAs (decitabine), thus blocking sAML transformation [26]. Together with *TP53*, mutations in *NRAS*/*KRAS*, *CEBPA*, and *FLT3* were observed in 63.6% of the 55 cases for which TRM were detected by targeted sequencing. When *RUNX1* /*CBL*/*PTPN11*/*WT1* and *ETV6* were included, only 10 mutated genes accounted for almost all the patients with MDS (94.5%) who experienced AML progression. In view of the rapid development of mutation-specific targeted therapy, we can hope to pharmacological block AML transformation in MDS patients at high transformation risk with regular monitoring for last mutations and administering effective corresponding targeted therapy.

In summary, somatic mutations in activated signaling, transcription factors, or tumor suppressor genes appear to be a precondition for AML transformation in myelodysplastic syndromes. The high propensity to acquire transformation-related gene mutations is worthy of further research as targets to be exploited with novel therapies.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81770120 and 81770122). We thank Shanghai Tianhao Inc. for providing assistance in exome sequencing and data analysis. We sincerely thank Dr. Rafael Bejar for providing constructive suggestion and revision.

Conflict of Interest

There are no financial and non-financial interests to declare.

Author contributions

X.L. was the principal investigators who conceived the study. C.-K.C., F.X., and L.-Y.W. carried out most of the experiments. L.-X.S.. and Q.H. were responsible for bioinformatics investigation. J.G. and Y.T. participated in the preparation of biological samples. Z.Z., D.W., L.-Y.Z., C.X. and J.-Y.S. helped gather detailed clinical information for the study and helped to carry out clinical analysis. X.L. , C.-K.C and F.X. wrote the manuscript.

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Figure 1 Occurrence frequency and sites of last mutation events

All mutations, including types and sites, are shown for 13 AML transformation-related mutations, according to the definitions. Triangles and circles indicate point and frameshift mutations, respectively. Stars indicates double mutations occurring in a single gene.

Figure 2 Evolution route for six cases that were analyzed by an additional sequencing assay between MDS diagnosis and AML transformation

Figure 3 Three evolution patterns for AML transformation

a. Linear evolution, based on the founding clone, is shown for UPN4687. Similar cases include UPN809, 1057, 3435, 3626, 1792, 3183, 1891, 1243, 3288, 1090, 1018, 3552, 3826, 4196, 4634, 1582, 1831, 2296, 843, 702, 3430, 3541, 2638, and 4133. **b**. Linear evolution from a subclone is shown for UPN2356. Similar cases include UPN4603, 2577, 692, and 2666. **c**. Sweeping clone evolution is shown for UPN2570. Similar cases include UPN811, 1354, and 3567. [Number] indicates VAF; [H] indicates homozygous mutations; red indicates presumed last mutation; green indicates newly emerged partner mutations, which could not be presumed to be AML transformation-related mutations.

Figure 4 Whole exome sequencing revealed other atypical last mutation events

Five patients showed negative results for typical AML transformation-related mutations. However, whole exome sequencing revealed some other atypical AML transformation-related mutations. Gray indicates existing mutations; red indicates activated signaling; blue indicates transcription factors; green indicates tumor suppressors.

Figure 5 Single-cell RNA transcription group sequencing revealed the linear association of gene mutations with activation of targeted signaling

(**a**) Patient presented with several abnormal cell groups (blue) after disease progression. (**b**) Cluster analysis suggested that abnormal GMP, CMP, MEP, and monocytes increased in the AML stage. (**c**) Integrated analysis indicated that several RAS signaling related-genes were highly expressed in GMP after disease progression. (**d**) RAS signaling related-genes, such as FLT3, INSR, and CDC42 were also highly expressed in MEP, CMP, and Mono groups. However, apoptosis-related gene BAD is down-regulated in GMP, MEP, and CMP.

Table.1 The results of target sequencing from paired samples of 64 patients

No (UPN)	Diagnosis	Duration (MDS to AML)(m)	Presumed last event	All gene mutations
The patients in whom AML transformation-related mutations could be presumed by paired sequencing analysis (listed as activated signaling, transcription factors and tumor suppressor Activated signaling				
1.(2998)	RAEB2		RUNX1/9/967+1>A6/886+1>A(31) KRAS/2/G12S(30)	ASXL1;KRAS;RUNX1;U2AF1
	AML	2	RUNX1/9/967+1>A6/886+1>A(28) ***KRAS/2/G12S(27)***	ASXL1;KRAS;RUNX1;U2AF1
2.(3430)	CMML1		GATA2/4/G327E(29)/4/323_323del(13) RUNX1/6/D198Y(41) KRAS/2/G12C(11) NRAS/2/G12D(29)	GATA2*2; KRAS;NRAS;RUNX1;SF3B1;UPF3A
	AML	7	GATA2/4/G327E(29)/4/323_323del(38) RUNX1/6/D198Y(43) ***KRAS/2/G12C(45)***	GATA2*2; KRAS; RUNX1;SF3B1;UPF3A; EZH2
3.（2356）	RAEB1		PHF6/4/C107fs(16) RUNX1/5/A149_A150delinsGX（16） TP53/3/c.74+14T>C(51)	BCOR;ITIH3;PHF6;RUNX1;TET2;TP53
	AML	6	PHF6/4/C107fs(23)	BCOR;ITIH3;PHF6;RUNX1;TET2;TP53；EZH2; KRAS

			RUNX1/5/A149_A150delinsGX（21） TP53/3/c.74+14T>C(55) ***KRAS/2/G13D(25)***	
4.（3541）	RAEB2		GATA2/5/L345W(43)	DNMT3A;GATA2;TET2
	AML	14	GATA2/5/L345W(30) ***KRAS/2/G12R（30）***	DNMT3A;GATA2;TET2;BCOR;KRAS
5.（809）	RCMD		CEBPA/1/K352fs（15） FLT3/9/D358V(48) RUNX1/6/508+1G>C(24)/5/G170R(23)	ASXL1;CEBPA;EZH2;FLT3;RUNX1*2;STAG2;TET2
	AML	17	FLT3/9/D358V(48) RUNX1/6/508+1G>C(45)/5/G170R(42) ***NRAS/2G13R(48)***	ASXL1; EZH2;FLT3;RUNX1×2;STAG2;TET2;BCOR; NRAS
6.（1057）	RAEB1		PTPRD/10/A539V（51）	DNMT3A; PTPRD;STAG2;U2AF1
	AML	48	PTPRD/10/A539V（46） RUNX1/4/L98fs(28) ***NRAS/2/3/G12V/Q61L(15)(15)*** ***CBL/8/C384R(12)***	DNMT3A; PTPRD;STAG2;U2AF1;CBL;NRAS×2;RUNX1
7.（3435）	RCMD		RUNX1/1/P86S(46)	ASXL1;EZH2;RUNX1;STAG2;UPF3A
	AML	33	RUNX1/1/P86S(43) PTPN11/3/F71L(34)	ASXL1;EZH2;RUNX1;STAG2;;UPF3A;BCOR;NRAS;PTPN11

			NRAS/2/G12D(9.7)	
8..(2747)	RAEB2			BCOR; ITIH3
	AML	10	***NRAS/2/G12D(57)***	BCOR; ITIH3;KIF20B;NRAS;STAG2;
9（4603）	RCMD		RUNX1/1/H78Y(53) ETV6/6/R339I(15)	DNMT3A;ETV6;EZH2;KIF20B;RUNX1;SETBP1
	AML	5	RUNX1/1/H78Y(43） ***NRAS/2/G12A(23)*** ***PTPN11/3/A72V(18)***	DNMT3A; KIF20B;RUNX1;SETBP1;NRAS;PTPN11;
10.（3626）	RCMD			ASXL1;DHX9;IDH1;RUNX1;TET2;U2AF1
	AML	29	ETV6/6/G381fs(48) ***NRAS/2/G12A(44)***	ASXL1;DHX9;IDH1;RUNX1;TET2;U2AF1;ETV6;NRAS
11.（4687）	RCMD		GATA2/3/M223I(51) RUNX1/2/G138fs(42)	ASXL1;EZH2;GATA2;RUNX1;STAG2;TET2*2
	AML	6	GATA2/3/M223I(55) RUNX1/2/G138fs(50) ***NRAS/2/G12D(52)***	ASXL1;EZH2;GATA2;RUNX1;STAG2;TET2*2;ROBO1;NRAS
12.(1587)	RAEB1		JAK2/14/V212F(46) FLT3/19/c.2208-14A.G(52)	IDH2;FLT3;JAK2;U2AF1;ZRSR2
	AML	25	JAK2/14/V212F(43) ***FLT3/19/c.2208-14A.G(49)***	IDH2;FLT3;JAK2;U2AF1;ZRSR2
13.(2570)	RAEB1		RUNX2/6/D198N(35)	IDH2;RUNX1;SRSF2
	AML	5	***FLT3/11/A445V(49)/11/A445S(49)***	FLT3*2;
14(1792)	RAEB1		RUNX1/9/Q397R（47）	RUNX1;U2AF1

	AML	8	RUNX1/9/Q397R（30） ***FLT3/11//E444V(37)11/E444X)(37)***	RUNX1;U2AF1; FLT3*2;ROBO1;
15.(2117)	RCMD		TP53/5/8/Y126C(16)/R273S(15)	SRSF2;TP53*2
	AML	8	TP53/5/8/Y126C(14)/R273S(14) ***FLT3/9/D358V(55)***	TP53*2;FLT3
16.(2577)	CMML1		CEBPA/1/p69-70del(17) NPM1/10/L258fs(33)	CEBPA;DNMT3A;NPM1;ROBO1;TET2
	AML	11	CEBPA/1/p69-70del(21) NPM1/10/L258fs(25) ***FLT3/14/K602delinsREYEYDLK (14)***	CEBPA;DNMT3A;NPM1;ROBO1;TET2;FLT3
17(3183)	RCMD			BCOR;DHX9;EZH2*2;IDH1;;ROBO1;STAG2;U2AF1
	AML	6	***FLT3/9/D358V(52)*** ***CBL/11/C1564-13C>T(40)***	BCOR;DHX9;EZH2*2;IDH1;ROBO1;STAG2;;U2AF1;CBL;FLT3;TET2
18 (2206)	RCMD		NF1/17/M645V(49) PTPRD/29A1047S(44) CBL/8/C396R(27）	ANKRD11;ASXL1;CBL;NF1;PTPRD;U2AF1
	AML	3	NF1/17/M645V(51) PTPRD/29A1047S(40) ***CBL/8/C396R(21)***	ANKRD11;ASXL1;CBL;NF1;PTPRD;U2AF1
19.(4559)	RAEB2		CBL/15/S791fs(9.6)/15/L790fs(9.7)	ANKRD11;ASXL1;CBL*2; ROBO1
	AML	11	***CBL/15/S791fs(8.7)/15/L790fs(8.8)***	ANKRD11;ASXL1;CBL*2; ROBO1
20.(692)	CMML2		GATA2/3/S186T(47)	GATA2;ITIH3;PHF6;RUNX1;SF3B1;

			PHF6/9/:c.968+1G>A（25） RUNX1/9/F416fs(27)	
	AML	10	GATA2/3/S186T(50) PHF6/9/:c.968+1G>A（26） RUNX1/9/F416fs(24) ***CBL/9/R420Q(34)***	GATA2;ITIH3;PHF6;RUNX1;SF3B1; CBL
21(1891)	CMML1			DNMT3A;ROBO2;TET2
	AML	14	***CBL/11/c.1564-13C>T(56)*** ***CEBPA/1/A265fs(37)K90fs(52)***	DNMT3A;ROBO2;TET2*2;CBL;CEBPA*2;EZH2
22.(4410)	RAEB1		PTPN11/3/D61H(33)	EZH2; PTPN11;SETBP1;TET2
	AML	12	***PTPN11/3/D61H(48)***	EZH2; PTPN11;SETBP1;TET2
23.(2638)	CMML1		PTPN11/14/P563R(43)	PTPN11; ROBO1;
	AML	16	***PTPN11/14//P563R(45) /3/D61V(37)***	PTPN11×2; ROBO1;SF3B1
24(4133)	RAEB1		CBL/9/R420Q(71) TP53/4/c.306-2A>G(96)	ASXL1;CBL;ROBO2;TP53
	AML	3	CBL/9/R420Q(39) TP53/4/c.306-2A>G(97) ***PTPN11/13/G507V(28)***	ASXL1;CBL;ROBO2;TP53;PTPN11
25.(3891)	RCMD		PTPRD/18/I930V（51）	ASXL1;PTPRD
	AML	8	***PTPRD/18/I930V(46)***	ASXL1;PTPRD
26.(702)	RCMD			ANKRD11;SRSF2

	AML	28	MPL/10/W515L(17) **GATA2/6/R396W(15)**	ANKRD11;SRSF2；GATA2;IDH1;MPL
Myeloid Transcriptors				
27 (1931)	RAEB1		***CEBPA/1/S9F(67)***	CEBPA;DNMT3A;TET2;U2AF1
	AML	9	***CEBPA/1/S9F(46)***	CEBPA;DNMT3A;TET2;U2AF1
28.(1243)	RCMD			ASXL1; U2AF1
	AML	18	***CEBPA/1/7_15del（40）***	ASXL1; U2AF1; CEBPA; IDH1
29.(3288)	RN			KIF20B；STAG2；TET2*2
	AML	12	***CEBPA/1/L338P(36)***	KIF20B；STAG2；TET2*2；ASXL1，CEBPA；EZH2
30.(1090)	RARS		RUNX1/9/D344G(45)	DNMT3A；DHX9；RUNX1； SRSF2；
	AML	11	RUNX1/9/D344G(48)/7/R232W(20) ***CEBPA/1/Y285fs(32)***	DNMT3A ;DHX9 ;RUNX1*2 ;SRSF2 ; ASXL1 ;CEBPA ;SETBP1
31.(1018)	RAEB2			DNMT3A; EZH2; TET2
	AML	4	FLT3:/9/D358V(53) ***CEBPA/1/A295V(39)***	DNMT3A; EZH2; TET2;BCOR;CEBPA；FLT3
32.(3552)	RARS			SF3B1; TET2
	AML	28	***CEBPA/1/c238delineCSG(18)***	SF3B1; TET2; CEBPA;; IDH2;SRSF2; STAG2
33(3826)	RCMD			ASXL1;EZH2
	AML	22	***CEBPA/1/Y166X(48)P58fs(47)***	ASXL1;EZH2;CEBPA*2
34.(4196)	RCMD		PHF6/4/G93S(27) RUNX1/1/R80C(51)	PHF6; RUNX1;SRSF2;

	AML	8	PHF6/4/G93S(45) RUNX1/1/R80C(78) NRAS/2/G12D（34） ***CEBPA/1/69_70del(11)***	PHF6; RUNX1;SRSF2; CEBPA;NRAS
35(4634)	RAEB2		NPM1/7/L160fs(24) NRAS/2/G13D(16)	NPM1；NRAS；SF3B1;TET2
	AML	2	NPM1/7/L160fs(35) NRAS/2/G13D(37) ***CEBPA/1/69_70del(9.8)***	NPM1；NRAS；SF3B1;TET2；CEBPA
36.(3536)	RCMD-RS		RUNX1/4/H105Y（40）	ASXL1;ROBO1;RUNX1;SF3B1;UPF3A
	AML	3	***RUNX1/4/H105Y（34）***	ASXL1;ROBO1;RUNX1;SF3B1;UPF3A
37.(1350)	RAEB1		PHF6/9/Y303X(56) RUNX1/9/P333fs(29)	EZH2;PHF6;RUNX1;TET2
	AML	2	PHF6/9/Y303X(46) ***RUNX1/9/P333fs(17)***	EZH2;PHF6;RUNX1;TET2
38(3479)	RCMD		RUNX1/3/R174Q(28)	ASXL1;RUNX1;TET2; ZRSR2
	AML	15	***RUNX1/3/R174Q(42)***	ASXL1;RUNX1;TET2*2;ZRSR2；
39.(4143)	RAEB1		RUNX1/4/S229fs（34）	BCOR;DNMT3A;IDH2;ROBO1;RUNX1
	AML	3	***RUNX1/4/S229fs（39）***	BCOR;DNMT3A;IDH2;ROBO1;RUNX1

40.(1582)	CMML1		PTPRD/22/c.962-9C>G(47)	KIF20B;PTPRD;SF3BA;TET2
	AML	13	PTPRD/22/c.962-9C>G(43) ***RUNX1/9/P340fs(40)***	KIF20B;PTPRD;SF3BA;TET2;RUNX1
41.(4390)	RCMD		ETV6/7/M389R(33) PHF6/10/R342X(27)	ASXL1;BCOR;ETV6;PHF6
	AML	15	PHF6/10/R342X(45) ***ETV6/7/M389R(42)***	BCOR;ETV6;PHF6
42.(811)	RCMD			None
	AML	26	***ETV6/6/V345L（52）*** ***MPL/10/W515R(50)***	ETV6;MPL
Tumor suppressors				
43.(1445)	RAEB2		TP53/8/E287D(56)	ROBO1;TP53;UPF3A
	AML	27	***TP53/8/E287D（48）***	ROBO1;TP53;UPF3A
44.(1694)	RAEB2		TP53/7/M237I(74)	DNMT3A; TP53
	AML	3	***TP53/7/M237I(80)***	DNMT3A; TP53
45.(3880)	CMML1		TP53/2/S83G(84)	TP53
	AML	13	***TP53/2/S83G(60)***	TP53
46(3678)	RAEB1		TP53/5/530-535del (34)	TP53
	AML	15	***TP53/5/530-535del（53）***	TP53
47.(1831)	RAEB1		TP53/4/W91X(30)	ANKRD11;DNMT3A;TP53
	AML	13	***TP53/4/W91X(30)/5/R158L(31)***	ANKRD11;DNMT3A;TP53*2;KIF20B;STAG2
48.(2666)	RAEB1		PTPRD/27/G809V(56)	DNMT3A;IDH2;PTPRD

	AML	18	PTPRD/27/G809V(51) ***TP53/5/P151S(25)***	DNMT3A;IDH2;PTPRD;TP53;
49(1354)	RAEB1			None
	AML	7	***TP53/8/E287fs(40)/6/G199E(28)***	ASXL1；TP53×2
50.(2296)	RAEB2			DHX9; ROBO1;
	AML	4.5	***TP53/9/R303fs(85)***	DHX9;ROBO1; TP53
51.(843)	RCMD			SRSF2; TET2
	AML	3	***TP53/5/C135X(18)***	SRSF2;TET2;TP53
52.(1849)	RCMD		WT1/7/H428fs（31）	DNMT3A;IDH1;TET2; WT1
	AML	8	***WT1/7/H428fs（40）***	DNMT3A;IDH1;TET2*2;WT1
53..(1670)	RCMD		GATA2/5/M388fs(41) TP53/2/E11Q(51) ETV6/5/V285M(49) WT1/1/A115fs(12)	BCOR;ETV6;GATA2;TET2;TP53;U2AF1；WT1
	AML	2	GATA2/5/M388fs(40) TP53/2/E11Q(50) ETV6/5/V285M(53) ***WT1/1/A115fs(25)***	BCOR;ETV6;GATA2;TET2;TP53;U2AF1；WT1
54(3567)	RAEB2		FLT3/12/c.1419-3->T(15) NPM1/10/R262fs(43)/10W259delinsW Q(44)/10/R262S(44)/7/W163L(42)	DHX9;DNMT3A;ANKRD11;FLT3; NPM*4;

	AML	4	NPM1/10/R262fs(12)/10W259delinsWQ(25)/10/R262S(19)/7/W163L(20) ***WT1/7/S169fs(32)***	DHX9;DNMT3A;ANKRD11; NPM×4;ROBO1;WT1
55.(1892)	RAEB2		NPM1/11/L287f（35）	DNMT3A;IDH1;NPM1
	AML	9	***NPM1/11/L287f（31）***	DNMT3A;IDH1;NPM1
The patients in whom last gene events could not be presumed by paired target sequencing analysis				
56(1075)	RA			DHX9;IDH2;ROBO2; STAG2;UPF3A
	AML	20		DHX9;IDH2;ROBO2; STAG2;UPF3A;TET2
57(1702)★	RAEB2			SRSF2
	AML	19		SRSF2
58.(1601)★	RAEB2			SETBP1
	AML	7.5		SETBP1
59.(3831)★	RAEB2			ANKRD11;IDH2
	AML	4		ANKRD11;IDH2
60.(3155)★	RCMD			ROBO1
	AML	7.5		ROBO1
61.(1619)	RAEB2			DHX9;DNMT3A;ITIH3*2
	AML	25		DHX9;DNMT3A;ITIH3*2
62.(3437)	RA			DNMT3A;ROBO1
	AML	3		DNMT3A;ROBO1

63.(3053) ★	RAEB2			STAG2(11);UPF3
	AML	40		SETBP1(18);SRSF2
64.(4145)	RCMD			EZH2;IDH1
	AML	14		ASXL1;TET2*2 ; ZRSR2

Notes:1. The fourth column indicates the possible last event mutations. The mutations with italic and bold font were shown as the final presumed AML transformation-related mutations (TRM) according to the definition in the METHOD part. 2. The paired target sequencing analysis from the last nine patients could not define the TRM, so five (with star signals)of them accepted the whole exome sequencing to look for possible TRM which were not included in 39 target genes.

Table 2. Characteristics summary for the detailed data from Table 1

Last event mutation	cases	Emerged at sAML(n)	Primary diagnosis≥ RAEB2/CMML2(n)	Most common earlier mutations at MDS diagnosis(top 2)(top4 in Total)(n)	Most common partner mutations at transformation(top 1)(top2 in Total)(n)
NRAS/KRAS	11(2)	9/11	3/11	RUNX1(8)/ASXL1(5)TET2(5)/EZH2(4) STAG2(4)	BCOR(3)
CEBPA	10(1)	9/10	2/10	TET2(6)/DNMT3A(4)/ASXL1(2)EZH2(2) SF3B1(2)RUNX1(2)SRSF2(2)U2AF1(2))	ASXL1(2)EZH2(2)
TP53	9	5/9	3/9	DNMT3A(3)/ROBO1(2)	ASXL1(1)KIF20B(1)Stag2(1)
FLT3	6(1)	5/6	0/6	U2AF1(3)/IDH2(2)RUNX1(2)SRSF2(2)	ROBO1(2)TET2(2)
CBL	6(3)	4/6	2/6	U2AF1(3)/ANKRD11(2)ASXL1(2)DNMT3A(2) STAG2(2)	TET2(2)
RUNX1	5	1/5	0/5	TET2(3)/ASXL1(2)SF3B1(2)	none
PTPN11	4(1)	3/4	0/4	EZH2(2)SETBP1(2)	SF3B1(1)
WT1	3	1/3	1/3	DNMT3A(2)TET2(2)	ROBO1(1)
Total	54(4)	37/54	11/54	TET2(16)/RUNX1(12)/ASXL1(11)DNMT3A(11)/EZH2(8)/U2AF1(8)	TET2(4)/ASXL1(3)BCOR(3)ROBO1(3)

NOTE: 1.The data from the cases with less observed TRM (less than 2 cases in total) is not included in this Table. 2. The number in the brackets of the second column represented the cases who had another presumed TRM besides the mainly presumed TRM. 3. The mutations listed in the last column means the mutations which emerged together with the presumed TRM, may playing roles during the MDS-sAML transformation.

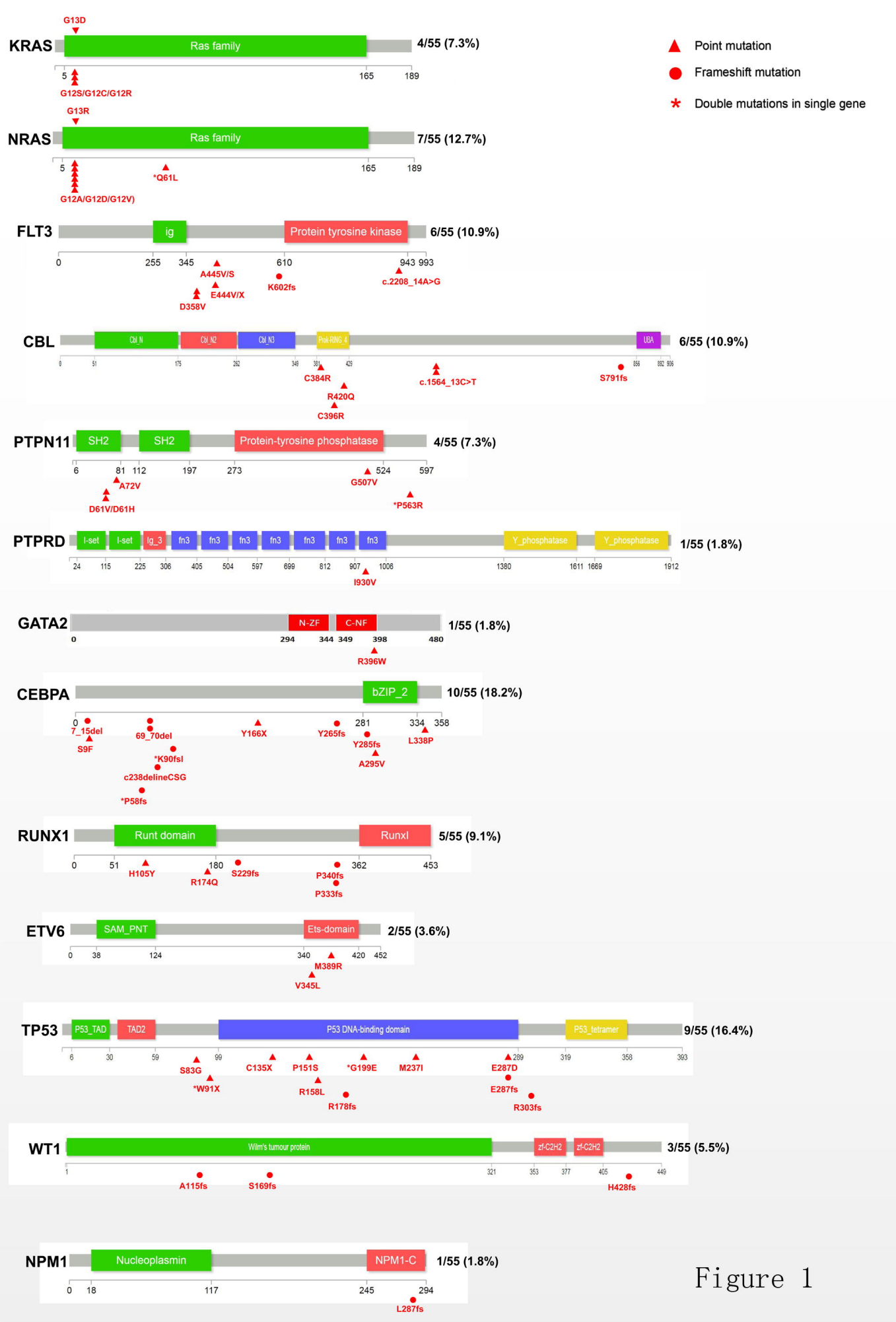
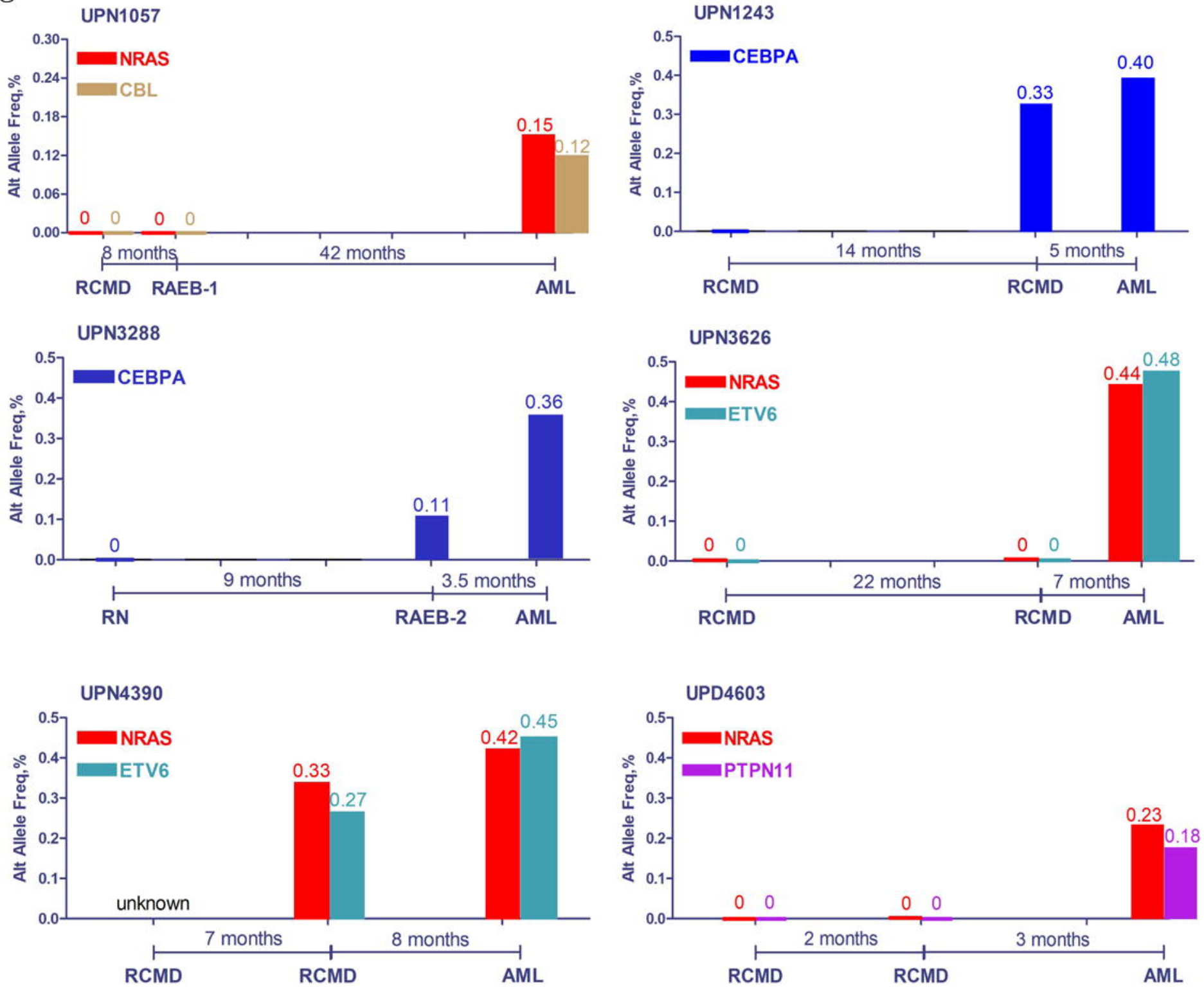

Point mutation
Frameshift mutation
Double mutations in single gene
KRAS
Ras family
4/55 (7.3%)
G13D
G12S/G12C/G12R
NRAS
Ras family
7/55 (12.7%)
G13R
*Q61L
G12A/G12D/G12V)
FLT3
ig
Protein tyrosine kinase
6/55 (10.9%)
A445V/S
K602fs
c.2208_14A>G
E444V/X
D358V
CBL
6/55 (10.9%)
C384R
R420Q
C396R
c.1564_13C>T
S791fs
PTPN11
SH2
SH2
Protein-tyrosine phosphatase
4/55 (7.3%)
A72V
D61V/D61H
G507V
*P563R
PTPRD
I-set
I-set
Ig_3
fn3
Y_phosphatase
Y_phosphatase
1/55 (1.8%)
I930V
GATA2
N-ZF
C-NF
1/55 (1.8%)
R396W
CEBPA
bZIP_2
10/55 (18.2%)
7_15del
S9F
69_70del
*K90fsI
c238delineCSG
*P58fs
Y166X
Y265fs
Y285fs
A295V
L338P
RUNX1
Runt domain
RunxI
5/55 (9.1%)
H105Y
R174Q
S229fs
P340fs
P333fs
ETV6
SAM_PNT
Ets-domain
2/55 (3.6%)
M389R
V345L
TP53
P53_TAD
TAD2
P53 DNA-binding domain
P53_tetramer
9/55 (16.4%)
S83G
*W91X
C135X
P151S
R158L
R178fs
*G199E
M237I
E287D
E287fs
R303fs
WT1
Wilm's tumour protein
zf-C2H2
zf-C2H2
3/55 (5.5%)
A115fs
S169fs
H428fs
NPM1
Nucleoplasmin
NPM1-C
1/55 (1.8%)
L287fs

Figure 1

Figure 2


UPN1057
NRAS
CBL
Alt Allele Freq,%
0.30
0.24
0.18
0.12
0.06
0.00
0
0
0
0
0.15
0.12
8 months
42 months
RCMD
RAEB-1
AML
UPN1243
CEBPA
0.5
0.4
0.3
0.2
0.1
0.0
0.33
0.40
14 months
5 months
RCMD
RCMD
AML
UPN3288
CEBPA
0
0.11
0.36
9 months
3.5 months
RN
RAEB-2
AML
UPN3626
NRAS
ETV6
0
0
0
0
0.44
0.48
22 months
7 months
RCMD
RCMD
AML
UPN4390
NRAS
ETV6
unknown
0.33
0.27
0.42
0.45
7 months
8 months
RCMD
RCMD
AML
UPD4603
NRAS
PTPN11
0
0
0
0
0.23
0.18
2 months
3 months
RCMD
RCMD
AML

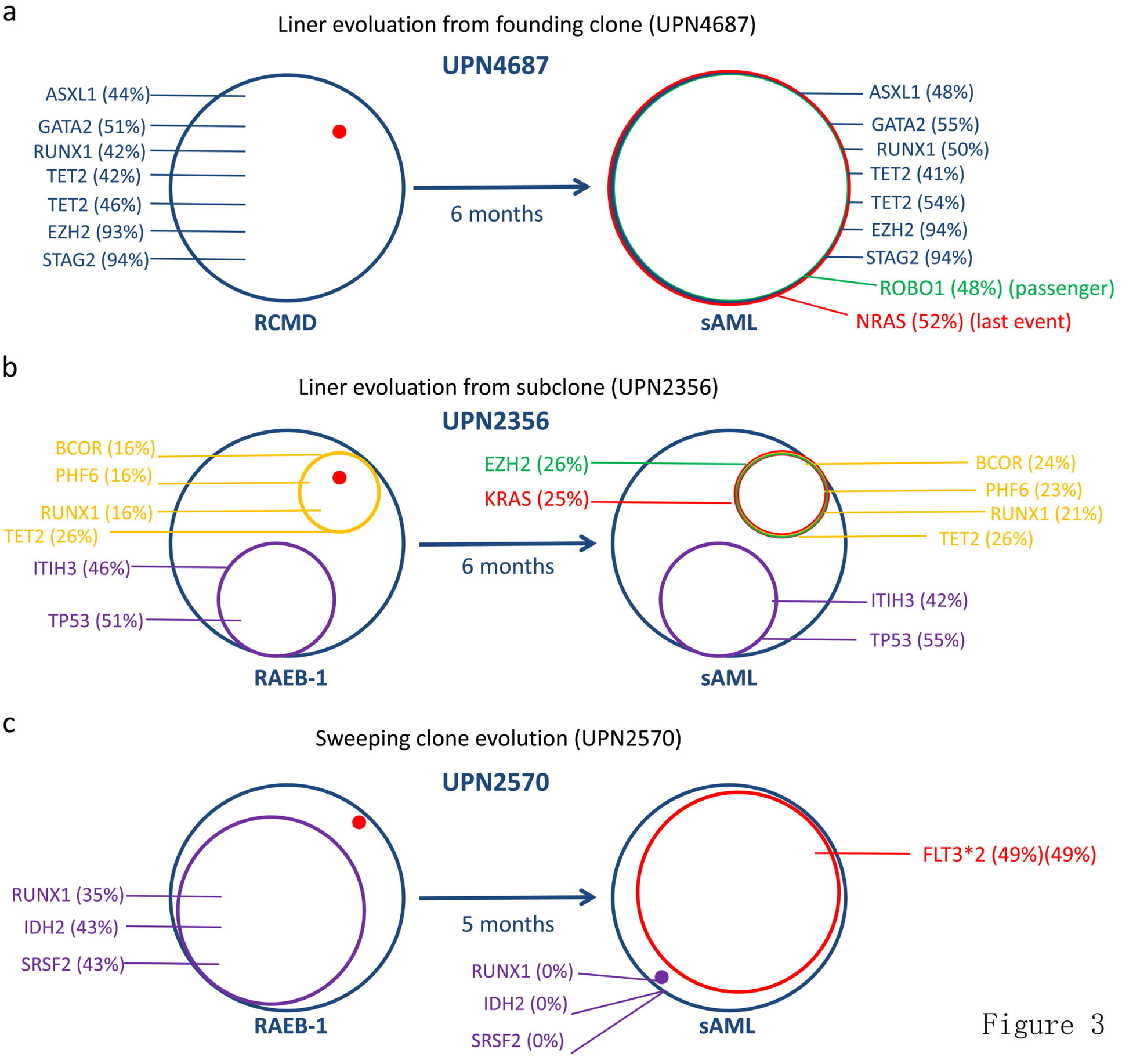

a
Liner evoluation from founding clone (UPN4687)
UPN4687
ASXL1 (44%)
GATA2 (51%)
RUNX1 (42%)
TET2 (42%)
TET2 (46%)
EZH2 (93%)
STAG2 (94%)
RCMD
6 months
ASXL1 (48%)
GATA2 (55%)
RUNX1 (50%)
TET2 (41%)
TET2 (54%)
EZH2 (94%)
STAG2 (94%)
ROBO1 (48%) (passenger)
NRAS (52%) (last event)
sAML
b
Liner evoluation from subclone (UPN2356)
UPN2356
BCOR (16%)
PHF6 (16%)
RUNX1 (16%)
TET2 (26%)
ITIH3 (46%)
TP53 (51%)
RAEB-1
6 months
EZH2 (26%)
KRAS (25%)
BCOR (24%)
PHF6 (23%)
RUNX1 (21%)
TET2 (26%)
ITIH3 (42%)
TP53 (55%)
sAML
c
Sweeping clone evolution (UPN2570)
UPN2570
RUNX1 (35%)
IDH2 (43%)
SRSF2 (43%)
RAEB-1
5 months
RUNX1 (0%)
IDH2 (0%)
SRSF2 (0%)
FLT3*2 (49%)(49%)
sAML

Figure 3

Figure 4

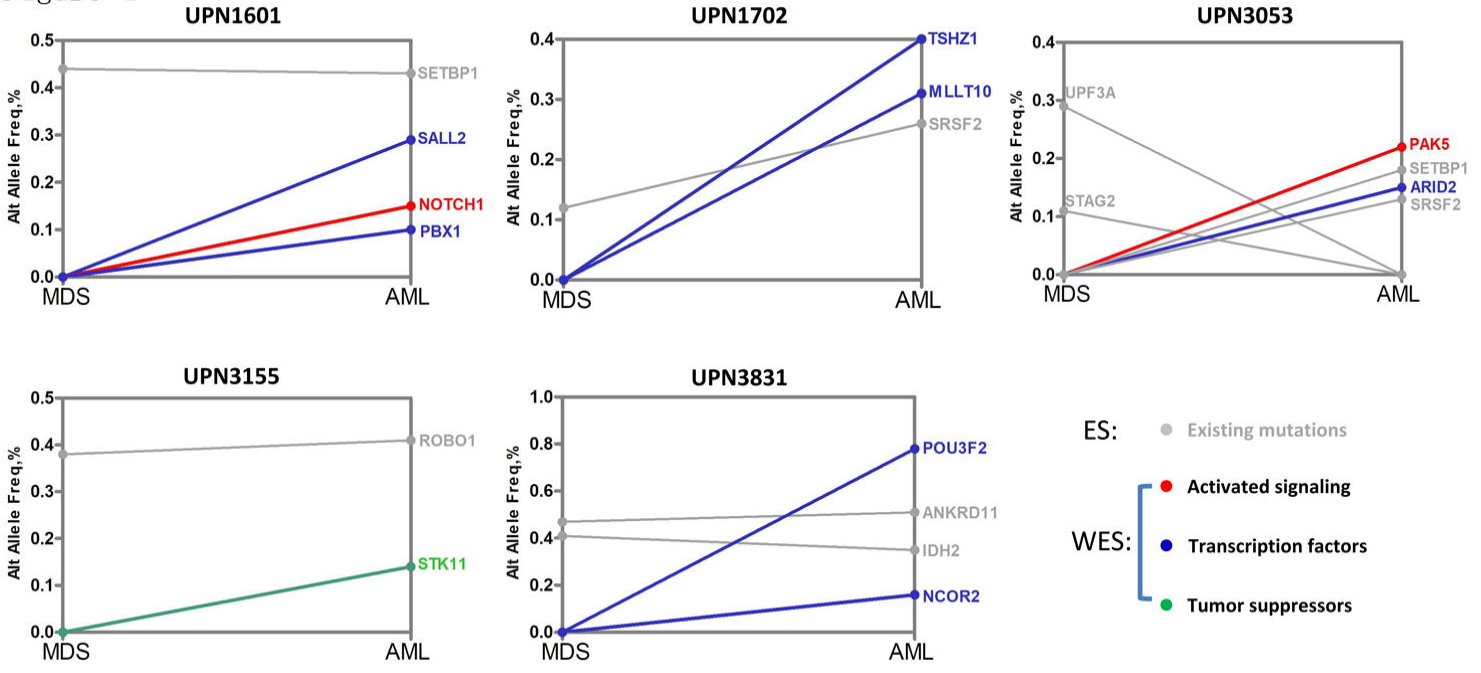
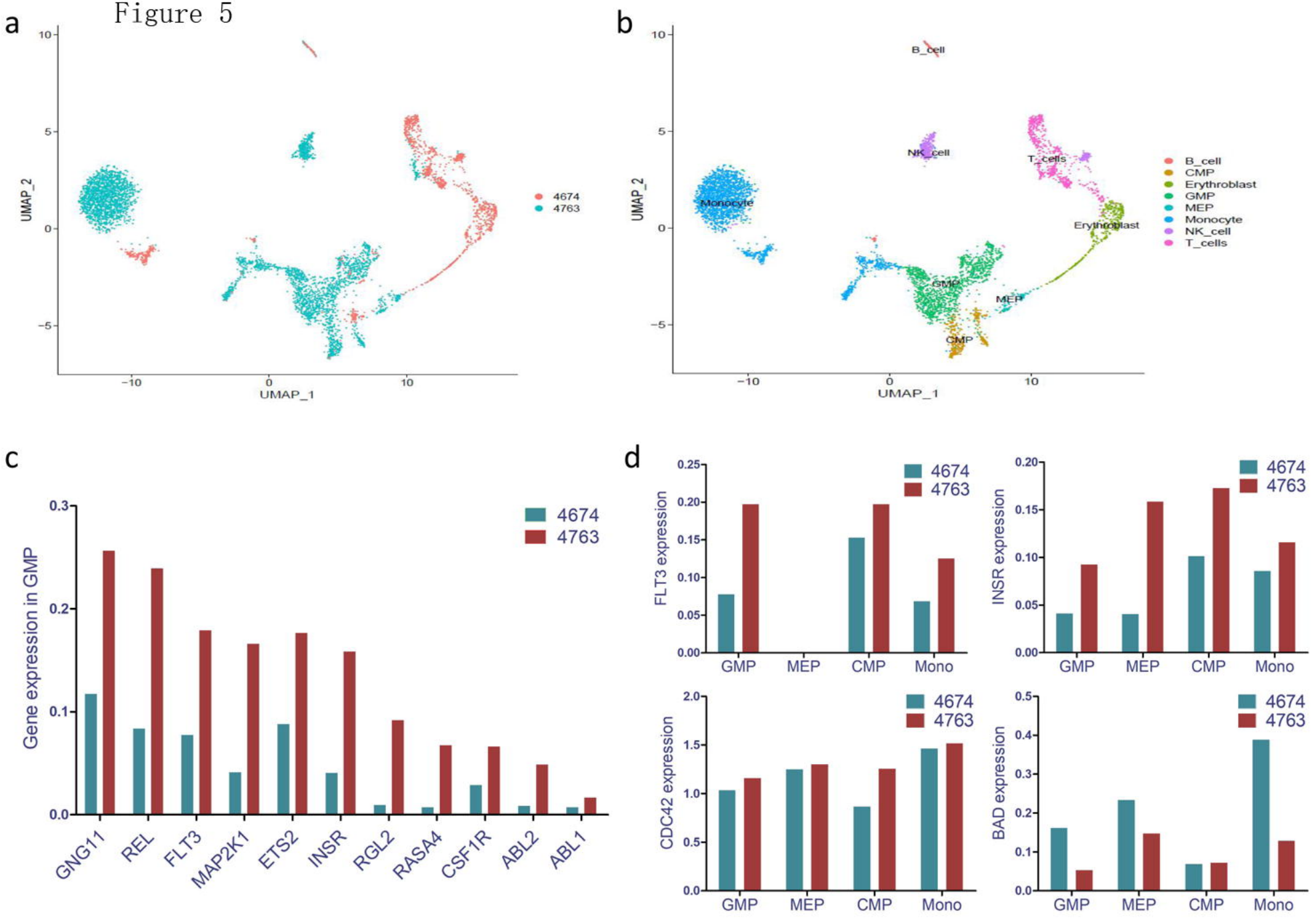

UPN1601
Alt Allele Freq,%
0.5
0.4
0.3
0.2
0.1
0.0
MDS
AML
SETBP1
SALL2
NOTCH1
PBX1
UPN1702
0.4
0.3
0.2
0.1
0.0
TSHZ1
MLLT10
SRSF2
UPN3053
UPF3A
STAG2
PAK5
SETBP1
ARID2
SRSF2
UPN3155
ROBO1
STK11
UPN3831
1.0
0.8
0.6
POU3F2
ANKRD11
IDH2
NCOR2
ES:
Existing mutations
WES:
Activated signaling
Transcription factors
Tumor suppressors

Figure 5


a
UMAP_2
UMAP_1
4674
4763
b
B_cell
NK_cell
T_cells
Monocyte
Erythroblast
GMP
MEP
CMP
B_cell
CMP
Erythroblast
GMP
MEP
Monocyte
NK_cell
T_cells
c
Gene expression in GMP
4674
4763
GNG11
REL
FLT3
MAP2K1
ETS2
INSR
RGL2
RASA4
CSF1R
ABL2
ABL1
d
FLT3 expression
INSR expression
CDC42 expression
BAD expression
GMP
MEP
CMP
Mono