Self-controlled Sequencing Suggests that Somatic Mutations of Signaling, Transcription and Tumor Suppression Genes are a Precondition for AML Transformation in MDS

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Research Article

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Abstract

Background

The transformation biology of secondary AML from MDS is still not fully understood. Here, we performed a large cohort of paired self-controlled sequences including target, whole-exome and single cell sequencing to search AML transformation-related mutations (TRMs).

Methods

39 target genes from paired samples from 72 patients with MDS who had undergone AML transformation were analyzed by next generation target sequencing. Whole exome and single-cell RNA sequencing were used to verify the dynamics of transformation.

Results

The target sequencing results showed that sixty-four out of the 72 (88.9%) patients presented presumptive TRMs involving activated signaling, transcription factors, or tumor suppressors. Of the 64 patients, most of TRMs (62.5%, 40 cases) emerged at the leukemia transformation point. All three of the remaining eight patients analyzed by paired whole exome sequencing showed TRMs which are not included in the reference targets. No patient with MDS developed into AML only by acquiring mutations involved in epigenetic modulation or RNA splicing. Single-cell sequencing in one pair sample indicated that the activated cell signaling route was related to TRMs which take place prior to phenotypic development. Of note, target sequencing defined TRMs were limited to a small set of seven genes (in the order: NRAS/KRAS, CEBPA, TP53, FLT3, CBL, PTPN11 and RUNX1, accounted for nearly 90.0% of the TRMs).

Conclusions

Somatic mutations involving in signaling, transcription factors, or tumor suppressors appeared to be a precondition for AML transformation from MDS. The TRMs may be considered as new therapy targets.

Introduction

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 special genetic abnormalities in different subsets [2–4]. 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 [5]. Unlike de novo AML, secondary AML (sAML), transformed from myelodysplastic syndromes (MDS), shows unique genetic features [6]. 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 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 still many unanswered questions in sAML transformation biology. Could early or late somatic mutations solely involved in epigenetic regulation or RNA splicing also induce sAML? 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? Answering of these questions will help us to find new insight of sAML pathogenesis, and then explore some new target therapy strategies. Logically, only systematized finding, but not some sporadic reports could answer these doubts. A systematized finding in this assay means the data should come from a big size sample, which could offer some frequency materials to focus our attention to some high occurrence rate mutations related to sAML transformations. In addition, a systematized finding should come from a paired self-controlled data, which would clearly tell us what mutations initiate MDS development, and what other mutations trigger off sAML transformation. Finally, the objects of observation in this study are focused on those mutated genes related to switch the sAML transformation, rather than an overall MDS clonal evolution [9]. In general, the functions of those mutations occur during early/middle stage of MDS (such as those MDS special gene mutations involving epigenetic regulation and RNA splicing) is to initiate clonal hematopoiesis and keep the phenotypes of MDS themselves. In theory, only those late events referring to signaling, transcription or tumor suppressor could start the sAML transformation process [7]. So the purpose of this study is to observe what happens on gene mutation when a MDS develops into sAML.

Paired self-controlled samples were acquired at MDS diagnosis and immediately after AML transformation. Sequencing for 39 target genes in all samples was followed by whole 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 some useful data that informs the biology of sAML transformation and suggests some novel strategies to block leukemic transformation of MDS.

Patients And Methods

Sample collection

From January 2004 until October 2020, a total of 1,541 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. Targeted sequencing was performed using paired

samples (at the MDS diagnosis and AML transformation points) to detect transformation-related gene mutations (TRMs). 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 were also included in this assay according to the FAB classification [12, 13]. Clinical and hematologic data were recorded following informed consent in accordance with the Declaration of Helsinki; this study was approved by hospital review boards of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

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, 98°C for 30s, 10 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 30 s, and 72°C 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*, *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 Wilcoxon 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 (TRMs): 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 (TRMs). Occasionally, over one mutation could be presumed as TRMs for one patient.

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 seventy-two MDS patients before and after transformation to sAML were analyzed using target sequencing. Identified somatic mutations are presented in Table 1. Of course, 72 cases was much less than actually sAML transformed patients number. In other word, because of loss of follow up and rejection of repeat sequencing, only a part of sAML patients were repeatedly sequenced and analyzed here.

1. Transformation-related mutations (TRMs) were identified in sixty-four of the 72 patients (88.9%), according to the steps described in METHODS.
2. Among these 64, TRMs were detected only at AML transformation in 40 patients (representing 62.5% of these cases). In the 24 remaining cases, these mutations were detected at the point of MDS diagnosis (Table 1). Patients with TRMs in only their sAML sample trended toward a longer time duration to undergo AML transformation (median 11.5 months vs. 8 months, $P=0.045$), but without significant difference in lower risk (<RAEB2/CMML2) cases frequency (77.5% vs. 75.0%, $P=0.819$).
3. The total defined TRMs number showed more than 64, because sometimes over one mutations could be considered as TRMs for one patient, and occasionally the TRMs for one case could be from both signaling and transcriptor. From Table 1, 2 and Figure 1, the following defined TRMs from the 64 patients were present in descending order of frequency. First were active signaling mutations: *KRAS/NRAS* in 14 patients; *FLT3* in 8 patients; *CBL* in 7 patients; *PTPN11* in 6, *PTPRD* in 2, *KIT* and *GATA2* in 1 respectively. The next set of mutations comprised myeloid transcription factors: *CEBPA* in 11 patients; *RUNX1* in 5 patients, *ETV6* in 3, and *SETBP1* in 2 patients respectively. The next set of mutations comprised tumor suppressor genes: *TP53* mutations in 9; *WT1* mutations in 3 patients, and *NPM1* in 1 patient. The TRMs events seemed to be highly enriched in 7 genes. Figure 1 demonstrated that the involvement style of *FLT3* (seven of the 8 cases appeared point mutations) was not the same as that of FLT3-ITD, which is more often observed in *de novo* AML.
4. In 6 of these 64 paired samples with candidate transformation events, we sequenced an additional sample between MDS diagnosis and AML transformation (Figure 2). Of these, 3 paired samples (UPN1243, 3288, and 4390) showed evidence of the TRMs emerging before phenotype change, i.e., the TRMs 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 64 patients with candidate mutation events, most (29 cases) demonstrated AML-transformation-related progression by linear evolution from the founding clone (Figure 3a). Five cases showed linear evolution from subclones (Figure 3b). Six cases demonstrated sweeping clonal evolution (Figure 3c). The evolution pattern of the remaining 24 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 TRMs were replaced or outgrown by other mutations at sAML transformation, such as in patient 1 (UPN809); 6 (UPN3430), 10 (UPN4603), 18 (UPN2570), and 63 (UPN3567) (Table.1)
7. Although differences existed among TRMs, TET2/RUNX1/ASXL1/ DNMT3A/U2AF1/STAG2/ROBO1/SF3B1 mutations were the most common initial/driver mutations for these AML-transformed patients (Table.2). However, ASXL1/BCORTET2/ mutations were most commonly accompanied by those transformation-related events when transformation occurred (Table.2).

Whole exome sequencing

Samples from three of the eight patients whose target sequencing showed no presumed TRMs 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 TRMs (involving transcription factor, or tumor suppressor genes) at the AML transformation point. Specifically, 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 [14]. NCOR2 regulates gene transcription as a part of a histone deacetylases complex, and its dysfunction leads to functional abnormality in hematopoietic stem cells [15]. 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 [16].

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 (*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 where 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 [17]. 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, which leads 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 [18]. Despite poor response to contemporary therapies, including stem-cell transplantation, 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 TRMs), and these factors are robust biomarkers of progression, it may be possible to develop target therapy.

In this study, we sequenced 72 paired bone marrow DNA samples, first at MDS diagnosis and then immediately after AML transformation. Sixty-four out of the 72 cases (88.9%) were positive for TRMs, i.e., events involving active signaling, transcription factors, or tumor suppressors were checked out by target sequencing. Sequencing of 39 target genes could not detect any TRMs in eight out of the 72 cases. Of these, paired whole exome sequencing was performed for 3 cases for which sufficient DNA could be extracted from BM samples. Each of these patients carried at least one TRM (out of the 39 target genes), involving myeloid transcription, or tumor suppressor genes (Figure 4). No patient with MDS in this assay 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 67 patients with detected TRMs, in 43 patients (64.2%; including 40 by targeted sequencing and 3 by WES) the TRMs appeared to emerge at the point of AML transformation, and the remaining 24 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 TRMs? Or the AML transformation itself. We considered that the pre-existence or emergence of TRMs should be the reason for AML transformation. First, for 24/67 (35.8%) cases harbored TRMs at MDS diagnosis, the duration of transformation was moderately shorter in these cases than in those where the TRMs were only identified at the transformation point (mean 8 vs. 11 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 TRMs 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 4674), 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 [19, 20]. Given that the gene mutations occurred prior to RNA/protein transcription/translation, pathway activation, and ultimately, phenotype alteration. These results support our findings that TRMs and activated *RAS* signaling can precede phenotypic transformation to AML.

Most of the TRMs clones showed linear evolution from the founding clones (29 cases, compared to 5 cases with linear evolution in a pre-existing subclone; and 6 cases by clone sweeping) (Figure 3). This is somewhat different from published data [21, 22], may because of that a part of patients did not presented clonal progressing process, such as those 24 cases whose TRMs pre-existed at MDS diagnosis. In addition, we detected common pre-existing mutations involving *TET2*, *RUNX1*, *ASXL1*, *DNMT3A*, *U2AF1*, *STAG2*, *ROBO1* and *SF3B1* (Table.2), indicating that these mutations represent precursor mutations for these sAML cases. We ever reported poor prognosis for those MDS with *ROBO* mutations [23]. In view of the non-signaling/transcriptor/tumor suppressor function of *ROBO*, which may assist TRMs to finish AML transformation. As for *ASXL1/BCOR/TET2* mutations, they emerged as common partners of TRMs (Table 2), possibly playing some roles in the transformation process. More research seemed necessary to explore the relationship between *BCOR* mutations (usually occur in patients with normal chromosomes) and sAML [24], especially for those sAML with *RAS* mutations as TRMs (Table.2). Some mutated genes such as *RUNX1* (highly prevalent among patients with chromosome 7 abnormalities) [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.

Together with *TP53*, mutations in *NRAS/KRAS*, *CEBPA*, *FLT3*, *CBL*, *PTPN11* and *RUNX1* (total 7 genes) accounted for almost 90% patients for whom TRMs were defined by targeted sequencing. *KIT* mutation was never detected as only TRM for one patient in this group of cases, 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]. Of note, we firstly reported *CEBPA* as one of the most common TRMs (accounting for nearly one-sixth of all TRMs), and expand the candidate genes for possible targeted treatment. In addition, the mutation pattern of *FLT3* in this assay less involved *FLT3-ITD* (only in one of the 8 cases) (often observed in *de novo* AML), proposing the necessity to design point-mutations-aimed target therapy for patients with this kind of TRMs. In view of the rapid development of mutation-specific targeted therapy in recent years, we can hope to pharmacological block AML transformation in MDS patients at high transformation risk with regular monitoring for TRMs 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.

Conclusion

We reported a large cohort of 72 cases of paired self-controlled data to identify the AML transformation-related mutations (TRMs). 88.9% of the 72 patients presented TRMs, which were mainly identified in a small set of genes including *RAS*, *CEBPA*, *TP53*, *FLT3*, etc. TRMs involving in signaling, transcription

factors, or tumor suppressors appeared to be a precondition for AML transformation from MDS. The TRMs may be considered as new therapy targets.

Declarations

Ethics approval and consent to participate

All procedures in the current study involving human samples were performed in accordance with the Helsinki declaration and approved by hospital review boards of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital. The informed consent has been obtained from each participant

Consent for publication

All authors have seen the manuscript and consented to publication.

Availability of data and materials

The data can be available by contacting the corresponding author.

Competing interests

There are no conflicts of interest to declare.

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Authors' contributions

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

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Tables

Table.1 The results of target sequencing from paired samples of 72 patients

No UPN	Diagnosis	chromo	(MDS to AML) (m)	Presumed last event	All gene mutations
<p><i>The patients in whom AML transformation-related mutations could be presumed by paired sequencing analysis (listed as activated signaling, transcription factors and tumor suppressor)</i></p> <p>Activated signaling</p>					
1.809	RCMD	normal		CEBPA/1/K352fs15 FLT3/9/D358V(48) RUNX1/6/508+1G>C(24)/5/G170R(23)	ASXL1;CEBPA;EZH2;
	AML	normal	17	FLT3/9/D358V(48) RUNX1/6/508+1G>C(45)/5/G170R(42) NRAS/2G13R(48)	ASXL1; EZH2;FLT3;R
2.1057	RAEB1	normal		PTPRD/10/A539V51	DNMT3A; PTPRD;ST
	AML	normal	48	PTPRD/10/A539V46 RUNX1/4/L98fs(28) NRAS/2/3/G12V/Q61L(15)(15) CBL/8/C384R(12)	DNMT3A; PTPRD;ST
3.2356	RAEB1	normal		PHF6/4/C107fs(16) RUNX1/5/A149_A150delinsGX16 TP53/3/c.74+14T>C(51)	BCOR;ITIH3;PHF6;RU
	AML	normal	6	PHF6/4/C107fs(23) RUNX1/5/A149_A150delinsGX21 TP53/3/c.74+14T>C(55) KRAS/2/G13D(25)	BCOR;ITIH3;PHF6;RU
4.(2998)	RAEB2	normal		RUNX1/9/967+1>A6/886+1>A(31) KRAS/2/G12S(30)	ASXL1;KRAS;RUNX1;
	AML	normal	2	RUNX1/9/967+1>A6/886+1>A(28) KRAS/2/G12S(27)	ASXL1;KRAS;RUNX1;
5. (2747)	RAEB2	normal			BCOR; ITIH3
	AML	normal	10	NRAS/2/G12D(57)	BCOR; ITIH3;KIF20B;l
6.(3430)	CMML1	normal		GATA2/4/G327E(29)/4/323_323del(13) RUNX1/6/D198Y(41) KRAS/2/G12C(11) NRAS/2/G12D(29)	GATA2*2; KRAS;NRA
	AML	normal	7	GATA2/4/G327E(29)/4/323_323del(38) RUNX1/6/D198Y(43) KRAS/2/G12C(45)	GATA2*2; KRAS; RUN
7.3435	RCMD	normal		RUNX1/1/P86S(46)	ASXL1;EZH2;RUNX1;
	AML	normal	33	RUNX1/1/P86S(43) PTPN11/3/F71L(34) NRAS/2/G12D(9.7)	ASXL1;EZH2;RUNX1;
8.3541	RAEB2	del20q-		GATA2/5/L345W(43)	DNMT3A;GATA2;TET
	AML	del20q-	14	GATA2/5/L345W(30) KRAS/2/G12R30	DNMT3A;GATA2;TET

9.3626	RCMD	normal			ASXL1;DHX9;IDH1;R
	AML	NA	29	ETV6/6/G381fs(48) NRAS/2/G12A(44)	ASXL1;DHX9;IDH1;R
10.4674	RCMD	Complex		RUNX1/1/H78Y(53) ETV6/6/R339I(15)	DNMT3A;ETV6;EZH2
	AML	Complex	5	RUNX1/1/H78Y(43) NRAS/2/G12A(23) PTPN11/3/A72V(18)	DNMT3A; KIF20B;RU
11.4687	RCMD	normal		GATA2/3/M223I(51) RUNX1/2/G138fs(42)	ASXL1;EZH2;GATA2;
	AML	NA	6	GATA2/3/M223I(55) RUNX1/2/G138fs(50) NRAS/2/G12D(52)	ASXL1;EZH2;GATA2;
12.(4877)	RCMD	normal		MPL/2/A58V(47) KRAS/2/G12S(41)	ASXL1;KRAS;MPL;S1
	AML	normal	17	MPL/2/A58V(46) KRAS/2/G12S(55) PTPRD/23/R588C(52)	ASXL1;KRAS;MPL;S1
13.(5305)	RCMD	normal		NRAS/2/G13D(4.1)/2/G12D(3.4)	IDH1;KMT2D;NRAS;
	AML	normal	9	NRAS/2/G13D(38) ETV6/4/H141Tfs313(16)	IDH2;KMT2D;NRAS;E
14.5253	RCMD	normal		None	None
	AML	t(3;6),-7	40	RUNX1/6/R201Q(48) NRAS/3/Q61H(25) PTPN11/3/E69K(23.4)	ANKRD11;NRAS;PTP
15.(1587)	RAEB1	der2		JAK2/14/V617F(46) FLT3/19/c.2208-14A.G(52)	IDH2;FLT3;JAK2;U2A
	AML	der2	25	JAK2/14/V617F(43) FLT3/19/c.2208-14A.G(49)	IDH2;FLT3;JAK2;U2A
16.(1792)	RAEB1	der4		RUNX1/9/Q397R47	RUNX1;U2AF1
	AML	der4	8	RUNX1/9/Q397R30 FLT3/11/E444V(37)11/E444X(37)	RUNX1;U2AF1; FLT3
17.(2117)	RCMD	complex		TP53/5/8/Y126C(16)/R273S(15)	SRSF2;TP53*2
	AML	complex	8	TP53/5/8/Y126C(14)/R273S(14) FLT3/9/D358V(55)	TP53*2;FLT3
18.(2570)	RAEB1	normal		RUNX2/6/D198N(35)	IDH2;RUNX1;SRSF2
	AML	+8	5	FLT3/11/A445V(49)/11/A445S(49)	FLT3*2;
19.(2577)	CMML1	normal		CEBPA/1/p69-70del(17) NPM1/10/L258fs(33)	CEBPA;DNMT3A;NPM
	AML	normal	11	CEBPA/1/p69-70del(21) NPM1/10/L258fs(25) FLT3/14/K602delinsREYEYDLK (14)	CEBPA;DNMT3A;NPM

20. (3183)	RCMD	add(3,+8)			BCOR;DHX9;EZH2*2;
	AML	add(3,+8)	6	FLT3/9/D358V(52) CBL/11/C1564-13C>T(40)	BCOR; DHX9; EZH2*; FLT3; TET2
21. (4383)	RCMD	normal			DNMT3A;SF3B1
	AML	+1,der(1;16)	18	PHF6/2/S22(5.6) FLT3/9/T382M(45) CBL/15/E765G(49) KIT/14/S713F(49)	DNMT3A;SF3B1;CBL
22. (5135)	RAEB2	Add7,del20q			ASXL1;ITIH3;TET2;U:
	AML	Add7,del20q	5	FLT3-ITDhigh1.597	ASXL1;ITIH3;TET2;U:
23.(692)	CMML2	normal		GATA2/3/S186T(47) PHF6/9/:c.968+1G>A(25) RUNX1/9/F416fs(27)	GATA2;ITIH3;PHF6;R
	AML	normal	10	GATA2/3/S186T(50) PHF6/9/:c.968+1G>A(26) RUNX1/9/F416fs(24) CBL/9/R420Q(34)	GATA2;ITIH3;PHF6;R
24. (1891)	CMML1	der22			DNMT3A;ROBO2;TE
	AML	der22	14	CBL/11/c.1564-13C>T(56) CEBPA/1/A265fs(37)K90fs(52)	DNMT3A;ROBO2;TE
25. (2206)	RCMD	+8		NF1/17/M645V(49) PTPRD/29A1047S(44) CBL/8/C396R(27)	ANKRD11;ASXL1;CB
	AML	+8	3	NF1/17/M645V(51) PTPRD/29A1047S(40) CBL/8/C396R(21)	ANKRD11;ASXL1;CB
26. (4559)	RAEB2	complex		CBL/15/S791fs(9.6)/15/L790fs(9.7)	ANKRD11;ASXL1;CB
	AML	complex	11	CBL/15/S791fs(8.7)/15/L790fs(8.8)	ANKRD11;ASXL1;CB
27. (2638)	CMML1	normal		PTPN11/14/P563R(43)	PTPN11; ROBO1;
	AML	normal	16	PTPN11/14//P563R(45) /3/D61V(37)	PTPN11×2; ROBO1;S
28. (4133)	RAEB1	complex		CBL/9/R420Q(71) TP53/4/c.306-2A>G(96)	ASXL1;CBL;ROBO2;T
	AML	complex	3	CBL/9/R420Q(39) TP53/4/c.306-2A>G(97) PTPN11/13/G507V(28)	ASXL1;CBL;ROBO2;T
29. (4410)	RAEB1	del20q		PTPN11/3/D61H(33)	EZH2; PTPN11;SETB
	AML	del20q	12	PTPN11/3/D61H(48)	EZH2; PTPN11;SETB
30 (4820)	RCMD	Add3,add18			EZH2;KMT2D;U2AF1
	AML	Add3	15	PTPN11/3/E76A(8.0)	EZH2;KMT2D;U2AF1
31. (3891)	RCMD	normal		PTPRD/18/I930V(51)	ASXL1;PTPRD

	AML	normal	8	<i>PTPRD/18/I930V(46)</i>	ASXL1;PTPRD
32.(702)	RCMD	der16,(1;16)del20q			ANKRD11;SRSF2
	AML	der16t(1;16)del20q	28	MPL/10/W515L(17) <i>GATA2/6/R396W(15)</i>	ANKRD11;SRSF2;GATA2
Myeloid Transcriptors					
33.(1018)	RAEB2	derX,der14			DNMT3A; EZH2; TET2
	AML	derX,der14	4	FLT3:/9/D358V(53) <i>CEBPA/1/A295V(39)</i>	DNMT3A; EZH2; TET2
34.(1090)	RARS	del20q		RUNX1/9/D344G(45)	DNMT3A;DHX9;RUNX1
	AML	del20q	11	RUNX1/9/D344G(48)/7/R232W(20) <i>CEBPA/1/Y285fs(32)</i>	DNMT3A;DHX9;RUNX1 SETBP1
35.(1243)	RCMD	normal			ASXL1; U2AF1
	AML	normal	18	<i>CEBPA/1/7_15del(40)</i>	ASXL1; U2AF1; CEBPA
36.(1931)	RAEB1	complex		<i>CEBPA/1/S9F(67)</i>	CEBPA;DNMT3A;TET2
	AML	complex	9	<i>CEBPA/1/S9F(46)</i>	CEBPA;DNMT3A;TET2
37.(3288)	RN	normal			KIF20B;STAG2;TET2
	AML	normal	12	<i>CEBPA/1/L338P(36)</i>	KIF20B;STAG2;TET2
38.(3552)	RARS	-Y			SF3B1; TET2
	AML	NA	28	<i>CEBPA/1/c238del(18)</i>	SF3B1; TET2; CEBPA
39.(3826)	RCMD	del7			ASXL1;EZH2
	AML	normal	22	<i>CEBPA/1/Y166X(48)P58fs(47)</i>	ASXL1;EZH2;CEBPA
40.(4196)	RCMD	del20q,+21		PHF6/4/G93S(27) RUNX1/1/R80C(51)	PHF6; RUNX1;SRSF2
	AML	del20q,+21	8	PHF6/4/G93S(45) RUNX1/1/R80C(78) NRAS/2/G12D(34) <i>CEBPA/1/69_70del(11)</i>	PHF6; RUNX1;SRSF2
41.(4634)	RAEB2	normal		NPM1/7/L160fs(24) NRAS/2/G13D(16)	NPM1;NRAS;SF3B1
	AML	normal	2	NPM1/7/L160fs(35) NRAS/2/G13D(37) <i>CEBPA/1/69_70del(9.8)</i>	NPM1;NRAS;SF3B1
42. 4806	CMML1	normal			ANKRD11;DHX9;TET2
	AML	+8	11	<i>CEBPA/1/1/E228fs(4.8)/L212Q(28)</i>	ANKRD11;DHX9;TET2
43.(1350)	RAEB1	complex		PHF6/9/Y303X(56) RUNX1/9/P333fs(29)	EZH2;PHF6;RUNX1;TET2
	AML	complex	2	PHF6/9/Y303X(46) <i>RUNX1/9/P333fs(17)</i>	EZH2;PHF6;RUNX1;TET2
44.(1582)	CMML1	normal		PTPRD/22/c.962-9C>G(47)	KIF20B;PTPRD;SF3B1
	AML	normal	13	PTPRD/22/c.962-9C>G(43)	KIF20B;PTPRD;SF3B1

<i>RUNX1/9/P340fs(40)</i>					
45. (3479)	RCMD	normal		RUNX1/3/R174Q(28)	ASXL1;RUNX1;TET2;
	AML	normal	15	<i>RUNX1/3/R174Q(42)</i>	ASXL1;RUNX1;TET2'
46. (3536)	RCMD-RS	normal		RUNX1/4/H105Y[40]	ASXL1;ROBO1;RUNX
	AML	del5q	3	<i>RUNX1/4/H105Y[34]</i>	ASXL1;ROBO1;RUNX
47. (4143)	RAEB1	normal		RUNX1/4/S229fs[34]	BCOR;DNMT3A;IDH2
	AML	normal	3	<i>RUNX1/4/S229fs[39]</i>	BCOR;DNMT3A;IDH2
48.(811)	RCMD	der6,t(1;6)			None
	AML	der6,t(1;6)	26	<i>ETV6/6/V345L[52]</i> <i>MPL/10/W515R(50)</i>	ETV6;MPL
49. (4390)	RCMD	normal		ETV6/7/M389R(33) PHF6/10/R342X(27)	ASXL1;BCOR;ETV6;P
	AML	inv3add3	15	PHF6/10/R342X(45) <i>ETV6/7/M389R(42)</i>	BCOR;ETV6;PHF6
50. (1601)	RAEB2	normal		SETBP1/3/c487-3C>T(44)	SETBP1
	AML	normal	8	<i>SETBP1/3/c487-3C>T(43)</i>	SETBP1
51. (3053)	RAEB2	normal			STAG2;UPF3A
	AML	normal	40	<i>SETBP1/4/D868N(18)</i>	SETBP1;SRSF2
Tumor suppressors					
52.(843)	RCMD	der1del1			SRSF2; TET2
	AML	der1	3	<i>TP53/5/C135X(18)</i>	SRSF2;TET2;TP53
53. (1354)	RAEB1	normal			None
	AML	complex	7	<i>TP53/8/E287fs(40)/6/G199E(28)</i>	ASXL1[TP53×2
54. (1445)	RAEB2	normal		TP53/8/E287D(56)	ROBO1;TP53;UPF3A
	AML	del11q	27	<i>TP53/8/E287D[48]</i>	ROBO1;TP53;UPF3A
55. (1694)	RAEB2	complex		TP53/7/M237I(74)	DNMT3A; TP53
	AML	complex	3	<i>TP53/7/M237I(80)</i>	DNMT3A; TP53
56. (1831)	RAEB1	complex		TP53/4/W91X(30)	ANKRD11;DNMT3A;T
	AML	complex	13	<i>TP53/4/W91X(30)/5/R158L(31)</i>	ANKRD11;DNMT3A;T
57. (2296)	RAEB2	normal			DHX9; ROBO1;
	AML	normal	4.5	<i>TP53/9/R303fs(85)</i>	DHX9;ROBO1; TP53
58. (2666)	RAEB1	normal		PTPRD/27/G809V(56)	DNMT3A;IDH2;PTPR
	AML	normal	18	PTPRD/27/G809V(51) <i>TP53/5/P151S(25)</i>	DNMT3A;IDH2;PTPR
59. (3678)	RAEB1	complex		TP53/5/530-535del(34)	TP53
	AML	complex	15	<i>TP53/5/530-535del[53]</i>	TP53
60. (3380)	CMML1	complex		TP53/2/S83G(84)	TP53
	AML	complex	13	<i>TP53/2/S83G(60)</i>	TP53

61. (1670)	RCMD	del18q		GATA2/5/M388fs(41) TP53/2/E11Q(51) ETV6/5/V285M(49) WT1/1/A115fs(12)	BCOR;ETV6;GATA2;T
	AML	del18q	2	GATA2/5/M388fs(40) TP53/2/E11Q(50) ETV6/5/V285M(53) <i>WT1/1/A115fs(25)</i>	BCOR;ETV6;GATA2;T
62. (1849)	RCMD	normal		WT1/7/H428fs[31]	DNMT3A;IDH1;TET2
	AML	normal	8	<i>WT1/7/H428fs[40]</i>	DNMT3A;IDH1;TET2
63. (3567)	RAEB2	normal		FLT3/12/c.1419-3>T(15) NPM1/10/R262fs(43)/10W259delinsWQ(44)/10/R262S(44)/7/W163L(42)	DHX9;DNMT3A;ANKK1
	AML	normal	4	NPM1/10/R262fs(12)/10W259delinsWQ(25)/10/R262S(19)/7/W163L(20) <i>WT1/7/S169fs(32)</i>	DHX9;DNMT3A;ANKK1
64. (1892)	RAEB2	normal		NPM1/11/L287f[35]	DNMT3A;IDH1;NPM1
	AML	normal	9	<i>NPM1/11/L287f[31]</i>	DNMT3A;IDH1;NPM1
<i>The patients in whom last gene events could not be presumed by paired target sequencing analysis</i>					
65. (1075)	RA	normal			DHX9;IDH2;ROBO2;S
	AML	normal	20		DHX9;IDH2;ROBO2;S
66. (1619)	RAEB2	complex			DHX9;DNMT3A;ITIH3
	AML	complex	25		DHX9;DNMT3A;ITIH3
67. (1702)[	RAEB2	normal			SRSF2
	AML	der12	19		SRSF2
68. (3831)[	RAEB2	normal			ANKRD11;IDH2
	AML	normal	4		ANKRD11;IDH2
69. (3155)[	RCMD	+14,del20q			ROBO1
	AML	normal	7.5		ROBO1
70. (3437)	RA	Inv9			DNMT3A;ROBO1
	AML	Inv9	3		DNMT3A;ROBO1
71. (4145)	RCMD	+1,der(1:7)			EZH2;IDH1
	AML	NA	14		ASXL1;TET2*2[ZRSI
72. [4404]	RAEB1	complex			SRSF2;TET2
	AML	complex	18		TET2

Notes:1. The fifth column indicates the possible last event mutations. The mutations with italic and bold font were shown as the final presumed AML transformation-related mutations (TRMs) according to the definition in the METHOD part. 2. The paired target sequencing analysis from the last eight patients could not define the TRMs, so three (with star signals) of them accepted the whole exome sequencing to look for possible TRMs which were not included in 39 target genes. For explanation of mutation type, splicing site mutation was shown in nucleotide variation. 3. NA means no available data.

Table 2. Characteristics summary of the top seven TRMs by target sequencing

TRMs	cases	Emerged at SAML(n)	Primary diagnosis≥ RAEB2/CMML2(n)	Most common earlier mutations at MDS diagnosis(top 2-3)(top5 in Total)(n)
<i>NRAS/KRAS</i>	14(5)	10/14	3/14	RUNX1(8)/ASXL1(6)TET2(6)STAG2(5)
<i>CEBPA</i>	11(1)	10/11	2/11	TET2(7)/DNMT3A(4)/ASXL1(2)EZH2(2)SF3B1(2)RUNX1(2)SRSF2(2)U2AF1(2)
<i>TP53</i>	9	5/9	3/9	DNMT3A(3)/ROBO1(2)
<i>FLT3</i>	8(2)	7/8	1/8	U2AF1(4)/IDH2(2)RUNX1(2)SRSF2(2)DNMT3A(2)ROBO1(2)TET2(2)
<i>CBL</i>	7(3)	5/7	2/7	DNMT3A(3)/U2AF1(3)/ANKRD11(2)ASXL1(2)STAG2(2)/PTPRD(2)/ROBO1(2)/SF3B1(2)
<i>PTPN11</i>	6(2)	4/6	0/6	EZH2(3)SETBP1(2)
<i>RUNX1</i>	5	1/5	0/5	TET2(3)/ASXL1(2)SF3B1(2)
<i>Total</i>	60(13)	42/60	11/60	TET2(18)/RUNX1(12)/ASXL1(12)DNMT3A(12)/U2AF1(9)/STAG2(7)ROBO1(6)SF3B1(6)

NOTE: 1.The TRMs (≤ 3 cases in total) are not included in this Table. 2. The number in the brackets of the second column represented the cases that had another presumed TRMs besides the mainly presumed TRMs. 3. The mutations listed in the last column means the mutations which emerged together with the presumed TRMs, may playing roles during the MDS-sAML transformation.

Figures

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 indicate 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

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, 4133, 4820, 4877, 5135, and 5305. **b.** Linear evolution from a subclone is shown for UPN2356. Similar cases include UPN4603, 2577, 692, and 2666. Liner evolution includes two models (from founding clone and subclone). Red dots mean the origin of red cycle (right graph). **c.** Sweeping clone evolution is shown for UPN2570. Similar cases include UPN811, 1354, 3053, 3567 and 4383. [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

Three 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; 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 (orange, 4674 before leukemia transformation and blue, 4763 after leukemia transformation). (b) Cluster analysis suggested that abnormal GMP, CMP, MEP, and monocytes increased in the AML stage. Cells are integrated and shown in two samples. Cells type are recognized and signed in different colors. (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.