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# Single cell genotyping of exome sequencing-identified mutations to characterize the clonal composition and evolution of inv(16) AML in a CBL mutated clonal hematopoiesis☆

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ABSTRACT

We recently described the development of an inv(16) acute myeloid leukemia (AML) in a *CBL* mutated clonal hematopoiesis. Here, we further characterized the clonal composition and evolution of the AML based on the genetic information from the bulk specimen and analyses of individual bone marrow cells for mutations in *CAND1*, *PTPRT*, and *DOCK6*. To control for allele dropout, heterozygous polymorphisms located close to the respective mutation loci were assessed in parallel. The clonal composition concluded from exome sequencing suggested a proliferation advantage associated with the acquisition of mutations in *CAND1*, *PTPRT*, and *DOCK6*. Out of 102 single cell sequencing reactions on these mutations and the respective polymorphisms, analyses yielded conclusive results for at least 2 mutation sites in 12 cells. The single cell genotyping not only confirmed the co-occurrence of the *PTPRT, CAND1* and *DOCK6* mutations in the same AML clone but also revealed a clonal hierarchy, as the *PTPRT* mutation was likely acquired after the *CAND1* and *DOCK6* mutations. This insight had not been possible based solely on the exome sequencing data and suggests that the mutation in *PTPRT*, which encodes a STAT3-inhibiting protein tyrosine phosphatase, contributed to the AML development at a later stage by enhancing proliferation.


## 1. Introduction

The knowledge about the clonal composition or architecture of acute myeloid leukemia (AML) allows conclusions regarding its pathogenesis. This is of particular interest in patients who have had a clonal hematopoiesis without an evident phenotype prior to development of the leukemia, i.e. clonal hematopoiesis of unknown significance or of indeterminate potential [1–4].

In recent years, next generation sequencing (NGS) has been increasingly used to gain more information about the clonal composition of hematologic malignancies as well as solid tumors. For this, the cell populations that share common mutations, i.e. clones, are identified from the variant allele frequencies (VAF) of the detected mutations. However, as this information is bioinformatically derived and might just yield an approximation of the definite clonal architecture at best, single cell genotyping is still required to verify the co-existence of mutations in a given cell and to derive reliable information about the clonal composition and evolution of a disease. Only few studies have yet investigated the clonal evolution of AML through the analyses of gene mutations in single cells [5–7], and, to our knowledge, no such study has yet been conducted in the context of a clonal hematopoiesis of indeterminate potential.

We recently described the first case of evolution of an inv(16) positive AML on a clonal hematopoiesis background due to a germline *CBL* mutation (defining the *CBL* syndrome) [8–10], and we identified possibly cooperating mutations by exome sequencing [11]. In the present study, we further characterized the clonal composition and evolution of the AML through the integrative analysis of the genetic data retrieved from the analysis of bulk specimens and those from single cell genotyping.

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## 2. Methods

### 2.1. Assessment of genetic aberrations in the bulk sample

Whole exome sequencing, CytoScan HD array (Affymetrix)-based assessment of copy number variations (CNV) and metaphase karyotyping of the bulk specimens were previously described; germ line or somatic origin of the gene mutations was verified in skin fibroblasts [11]. Written informed consent was obtained prior to sampling. The AML cell line Kasumi-1 was analyzed for *KIT* exon 17 mutations as previously reported [12], and by the Human SNP Array 6.0 (Affymetrix) according to the manufacturer's recommendations.

### 2.2. Single cell isolation and whole genome amplification

For the isolation of single cells, cryo-conserved specimens of the Kasumi-1 cell line or of Ficoll-enriched bone marrow aspirates were thawed in pre-warmed PBS (37 °C) and washed twice with PBS at 4 °C. The cells were DAPI stained by incubation in DAPI working solution for 10 min at room temperature. DAPI negative, single cells were placed into each well of a PCR plate using a MoFlo high speed cell sorter (Beckman Coulter). Genomic DNA was amplified by whole genome amplification (WGA) using the REPLI-g Mini Kit (Qiagen), which is based on a multiple displacement amplification and results in DNA fragments with a length of more than 10 kb. The WGA was performed according to a modified protocol. Briefly, 0.75 μl buffer D2 were added into each well, the plate was centrifuged (1000*g*), gently mixed by vortexing and again briefly centrifuged. For cell lysis and DNA denaturation, the plate was incubated for 10 min at 65 °C, followed by immediate cooling on ice and addition of 0.75 μl stop solution. To each well, 10 μl of a master mix containing 2.25 μl nuclease-free water, 7.25 μl REPLI-g mini reaction buffer and 0.5 μl Repli-g mini DNA polymerase were added, and the plate was incubated at 30 °C for 8 h. A multiplex PCR on repetitive *LINE1* retrotransposons was used in a portion of single cells in order to evaluate the success of the WGA.

### 2.3. Single cell genetic analyses

The amplified DNA of each cell was subjected to PCR and Sanger sequencing of the respective mutation loci. The WGA can lead to allele dropout (ADO) and thus to false results when heterozygous mutations are analyzed and only the wild-type allele is amplified. To control for ADO we also sequenced single nucleotide polymorphisms (SNPs), which had been found to be heterozygous in the sample by the CytoScan HD or SNP 6.0 array and that were located nearby the respective mutation loci. The heterozygosity of each SNP was verified by Sanger sequencing in the bulk sample. If, in a single cell, there was no mutated sequence at the mutation site and the respective SNP not heterozygous, then ADO may have occurred at the genomic locus, and the mutation analysis was deemed to be inconclusive.

Primer sequences are provided in the supplemental material.

## 3. Results

### 3.1. Genetic aberrations in AML and estimation of the proportion of affected cells

We first performed an integrative analysis of the genetic data retrieved from the chromosome banding, microarrays and exome sequencing experiments in order to estimate the fraction of AML cells in the bone marrow which harbored a specific aberration (Table 1). Based on the detection of the inv(16) in all 22 metaphases analyzed, we concluded that inv(16) was present in all cells. The microarray patterns showing loss of heterozygosity of chromosome 11q (LOH 11q) and the additional gain of 11q material in the absence of any heterozygous signal at this position suggested that the 11q aberrations were also present in all cells. Moreover, the absence of other chromosomal aberrations in the microarray suggested that all the gene mutations detected by exome sequencing were present in a heterozygous state. Based on this and the VAFs, we calculated the proportion of cells harboring the respective gene mutations.

**Table 1**
Genetic aberrations and conclusions about the proportion of cells harboring the respective aberration.

| Gene | Mutation | VAF | Zygosity[a] | Proportion of cells |
|---|---|---|---|---|
| *CBFB,MYH11* | inv(16) [22] | n.a. | n.a. | 100% |
| *CBL* | p.D390V | 100% | LOH11q/gain11q | 100% |
| *KIF14* | p.V341I | 51% | heterozygous | 100% |
| *TMEM125* | p.D113N | 51% | heterozygous | 100% |
| *MIOX* | p.W225R | 46% | heterozygous | 92% |
| ***CAND1*** | p.E584* | 39% | heterozygous | 78% |
| *NID2* | p.D319N | 38% | heterozygous | 76% |
| *ARF3* | p.N101S | 36% | heterozygous | 72% |
| *PRSS16* | p.R491C | 36% | heterozygous | 72% |
| ***PTPRT*** | p.T844M | 33% | heterozygous | 66% |
| ***DOCK6*** | p.R1872_K1873insP | 33% | heterozygous | 66% |
| *ADAM12* | p.A222V | 21% | heterozygous | 42% |
| *CMIP* | p.T323M | 15% | heterozygous | 30% |
| *MYOCD* | p.D283N | 7% | heterozygous | 14% |

[a] Zygosity deduced from the copy number variation microarray. VAF, variant allele frequency; n.a., not applicable; LOH, loss of heterozygosity.

### 3.2. Clonal evolution of the AML based on the genetic aberrations

For the derivation of the potential clonal evolution of the AML, we assumed a linear pattern, i.e. all aberrations were acquired within a single lineage. The sequence of the acquisitions was determined based on the estimated proportion of cells affected by each of the aberrations, in such a way that mutations present in a smaller subset of cells were acquired after those present in a larger subset of cells.

As previously described [11], the germ line *CBL* mutation and the additionally acquired loss of the remaining *CBL* wild-type allele through the LOH 11q resulted in a clonal hematopoiesis. Towards AML, inv(16), gain 11q and the mutations identified by exome sequencing were acquired (Fig. 1A). Among these mutations were several with similar VAFs (Table 1). Since small differences between the VAFs may be due to technical reasons and, in turn, the estimates of cells harboring a specific mutation inaccurate, we created three mutation groups: Group I comprised the mutations in *KIF14*, *TMEM125*, and *MIOX* (proportion of cells, 92–100%), group II the mutations in *CAND1*, *NID2*, *ARF3*, *PRSS16*, *PTPRT*, and *DOCK6* (66–78%), and group III, the mutations in *ADAM12*, *CMIP*, and *MYOCD* (14–42%) (Fig. 1A).

We assumed that these groups also represent steps during disease evolution: Group I mutations were gained during the AML initiating event. Group II mutations were acquired later, but appeared to have accelerated the proliferation (Fig. 1B). We hypothesized that this acceleration was caused by the mutation, which was gained last within group II. Since this information could not be retrieved from the exome sequencing data, we sought to assess the mutation status at the single cell level. For this, we focused on *CAND1*, *PTPRT*, and *DOCK6*; mutations in these genes had been previously described in AML and were likely deleterious.