Targeted resequencing analysis of 31 genes commonly mutated in myeloid disorders in serial samples from myelodysplastic syndrome patients showing disease progression

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The myelodysplastic syndromes (MDS) are clonal disorders of the hematopoietic stem cell, characterized by ineffective hematopoi-

esis and peripheral blood cytopenias, and patients typically have a hypercellular bone marrow. Approximately 30–40% of patients undergo leukemic transformation to acute myeloid leukemia (AML) during the course of their disease.1 Several recurrent gene mutations have been identified in MDS using next-generation sequencing (NGS), and recent studies have greatly illuminated the molecular landscape of this disorder.2–4 However, the molecular events driving MDS progression to AML remain poorly understood.

To investigate the genetic basis of disease progression in MDS, and in particular of leukemic transformation to AML, we evaluated the frequency and chronology of the acquisition of a large number of gene mutations using a targeted NGS myeloid gene panel on serial (paired) bone marrow samples from 41 MDS patients before (preprogression) and after progression (postprogression) to a more advanced subtype (n = 5) or to AML (n = 36) (Supplementary Table 1). The mutational profile was characterized using a TruSeq Custom Amplicon (TSCA) panel (Illumina, San Diego, CA, USA), a development of our previously reported myeloid gene panel,5 targeting the hotspots of 31 recurrently (>1%) mutated genes in myeloid malignancies (Supplementary Table 2). Dual-barcoded TSCA libraries were sequenced on an Illumina MiSeq platform, and variants were annotated and filtered using Illumina VariantStudio (Supplementary Methods). The proportion of sequencing reads supporting a given mutation (variant allele frequency, VAF) was used to estimate the fraction of tumor cells carrying that mutation, and to determine whether mutations are clonal (in all tumor cells) or subclonal (in a fraction of tumor cells).

A total of 99 and 122 mutations across 23 genes were identified in preprogression and postprogression samples, respectively (Supplementary Tables 1 and 3). The number of mutations was generally higher in the postprogression samples: the number of cases with one or two mutations was 24 in preprogression samples and 17 in postprogression samples, whereas the number of cases with three or four mutations was 12 in preprogression samples and 17 in postprogression samples (Supplementary Figure 1). These data are consistent with a previous study showing that, in a large MDS patient cohort, leukemia-free survival deteriorated steadily as the number of driver mutations increased, suggesting that transformation to AML is driven by clonal evolution associated with the acquisition of new driver mutations.3

The most frequently mutated genes (in >15% of samples) were ASXL1, TET2, SRSF2, U2AF1, RUNX1 and TP53. ASXL1, encoding an...
epigenetic regulator, was the top ranking mutated gene with a frequency of 44% in preprogression samples and 46% in postprogression samples (Table 1). In contrast, the splicing factor SF3B1, widely reported as the most frequently mutated gene in MDS, was mutated in only two cases in our cohort (5%). Given that the frequency of ASXL1 mutations in MDS ranges from 11% to 15%, and of SF3B1 mutations from 20% to 28% in unselected studies, the mutation data concerning these two genes are clearly strikingly different in our study. It should be noted, however, that the patient cohort used in this present study was highly selected—that is, comprising only patients whose disease had progressed. Our data thus indicate that ASXL1 mutations are strongly associated with MDS cases that show disease progression to AML and conversely that ASXL1 is a poor prognostic marker in MDS7 and with the strong association of SF3B1 mutation with a good prognosis in MDS and with the low-risk MDS subtype refractory anemia with ring sideroblasts.8

In agreement with previous studies,2,3,9 mutations in splicing factor genes (SF3B1, SRSF2, U2AF1 and ZRSR2) were mutually exclusive, with the only exception being one case with mutations in both U2AF1 and ZRSR2. Mutations of genes involved in splicing (SRSF2, U2AF1 and ZRSR2), chromatin modification (EZH2 and ASXL1) and DNA methylation (TET2, IDH1/2 and DNMT3A) were present in the preprogression and postprogression samples for almost all cases harboring mutations in these genes, and thus represent early events in the disease course in these cases. Interestingly, age-related clonal hematopoiesis, with the majority of variants occurring in DNMT3A, TET2 and ASXL1, has been shown to be a common condition that is associated with increases in the risk of hematologic cancer.10,11 Mutations of genes involved in transcriptional regulation (RUNX1, ETV6 and PHF6) and signal transduction (NRAS and KRAS) were found in the postprogression sample only in the majority of cases, suggesting that these are often late events that may co-operate with early events to drive disease progression (Figure 1). Papaemmanuil et al.12 similarly showed that mutations in genes involved in RNA splicing and DNA methylation occur early during the disease course in MDS, whereas driver mutations in genes involved in signaling often occur later. However, this study concemed the analysis of different MDS subtypes, not serial samples from the same patients. Emerging data suggest that key differences in disease phenotype can be driven by different combinations of comutated genes in MDS.12 Reasoning that such gene mutation associations may also have a role in disease progression in MDS, we investigated pairwise associations between mutated genes in our study of MDS serial samples. We found that all seven preprogression samples carrying RUNX1 mutations were also mutated for ASXL1, whereas 11 of 34 preprogression samples without RUNX1 mutations had ASXL1 mutations (two-sided \( P = 0.001 \), Fisher’s exact test). The ASXL1–RUNX1 mutated gene association has been previously shown to be significant in studies on large MDS cohorts.2,3 We also found that all five preprogression samples in our cohort with ZRSR2 mutations also carried ASXL1 mutations, whereas 13 of 36 preprogression samples without ZRSR2 mutations carried ASXL1 mutations (two-sided \( P = 0.011 \), Fisher’s exact test). Co-occurrence of mutations in splicing factor genes and in genes involved in epigenetic regulation has been reported previously,3,9 however, the ASXL1–ZRSR2 association has not been described. In the postprogression samples, there were five cases with mutations of both NRAS and RUNX1 (compared with two cases in preprogression samples), an association reported as significant in a previous MDS study.3 We have also observed co-occurrence of NRAS and ASXL1 mutations in five postprogression samples (compared with two cases in preprogression samples) in our study. Interestingly, NRas and Asxl1 loss co-operate to drive myeloid proliferation and myeloid leukemia in mice,13 and our data on MDS serial samples support this observation. These co-occurring gene mutations may thus have a role in disease progression in MDS.

Specific associations between mutated genes and chromosomal abnormalities have also been described in MDS.6 In our study, we found that TP53 mutations were present in all four cases in our cohort with abnormalities of chromosome 5, U2AF1 mutations were present in three of five cases with –20/del(20q) and two of four cases carrying SETBP1 mutations had –7; these association have been previously reported.6

The average VAF of some mutations changed markedly during disease progression, with TP53 showing the largest average VAF fold increase (>50%) in postprogression samples compared with preprogression samples among genes mutated in more than five cases, suggesting that this mutation had a major role in driving disease progression in these patients (Supplementary Table 4). RUNX1 showed an average VAF fold increase of approximately 25% in postprogression samples compared with preprogression samples, and interestingly both RUNX1 and TP53 mutations are also strongly associated with a poor prognosis in MDS.7 Conversely, the average VAF of TET2, ZRSR2, EZH2 and U2AF1 was similar between preprogression and postprogression samples (Supplementary Table 4). The VAF of a small number of mutations decreased in the postprogression samples, possibly owing to the dominant clone not being ancestral or to the emergence of competing subclones carrying mutations that were not detected by our gene panel.

The large majority of TET2, ZRSR2 and EZH2 mutations in preprogression samples had VAFs >40%, within a range that, according to previous studies that analyzed the clonal architecture of MDS and AML from WES or WGS data,14,15 is consistent with these mutations being present in a founding clone. Six out of eight TP53 mutations found in preprogression samples had VAFs smaller than 30% (range 8–27%), suggesting that mutations of this gene occurred mainly in a subclone; four of these subclonal mutations expanded with disease progression and two additional TP53 mutations were present in postprogression samples only. Four NRAS mutations identified in preprogression samples were

### Table 1. Number and percentage of mutated preprogression and postprogression samples for each gene

| Genes   | Number (%) of mutated preprogression samples | Number (%) of mutated postprogression samples |
|---------|----------------------------------------------|----------------------------------------------|
| ASXL1  | 18 (44%)                                     | 19 (46%)                                     |
| TET2   | 11 (27%)                                     | 11 (27%)                                     |
| U2AF1  | 10 (24%)                                     | 8 (20%)                                      |
| RUNX1  | 7 (17%)                                      | 11 (27%)                                     |
| TP53   | 7 (17%)                                      | 17 (41%)                                     |
| SRSF2  | 6 (15%)                                      | 7 (17%)                                      |
| EZH2   | 5 (12%)                                      | 5 (12%)                                      |
| ZRSR2  | 5 (12%)                                      | 6 (15%)                                      |
| IDH2   | 4 (10%)                                      | 4 (10%)                                      |
| NRAS   | 3 (7%)                                       | 8 (20%)                                      |
| IDH1   | 3 (7%)                                       | 3 (7%)                                       |
| SETBP1 | 2 (5%)                                       | 4 (10%)                                      |
| PHF6   | 2 (5%)                                       | 3 (7%)                                       |
| DNMT3A | 2 (5%)                                       | 2 (5%)                                       |
| CBL    | 1 (2%)                                       | 1 (2%)                                       |
| KIT    | 1 (2%)                                       | 1 (2%)                                       |
| CSE3R  | 1 (2%)                                       | 0 (0%)                                       |
| ETV6   | 1 (2%)                                       | 4 (10%)                                      |
| SF3B1  | 1 (2%)                                       | 2 (5%)                                       |
| NPM1   | 1 (2%)                                       | 1 (2%)                                       |
| ATRX   | 0 (0%)                                       | 2 (5%)                                       |
| KRAS   | 0 (0%)                                       | 1 (2%)                                       |
| FLT3   | 0 (0%)                                       | 2 (5%)                                       |

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subclonal (VAF range 7–30%); eight NRAS mutations were present in postprogression samples only, suggesting that the emergence of new NRAS mutations during the course of the disease may have a role in disease progression. The VAF of all three subclonal mutations in RUNX1 in preprogression samples (VAF range 12–19%) increased with disease progression; in addition, four RUNX1 mutations were found in postprogression samples only, suggesting that in the case of RUNX1 both the emergence of new mutations and the expansion of existing ones during the disease course may be involved in progression to AML.

An additional serial sample was sequenced for four cases in our cohort (Supplementary Figure 2). In one case, an NRAS mutation expanded during disease progression, whereas the allele burden of mutations in ASXL1, RUNX1 and EZH2 remained constant. In another case, within a background of mutations in ASXL1, EZH2 and ZRSR2, a mutation in SETBP1 emerged mid-progression and a mutation in NRAS was found at the AML stage only. This shows that more precise information on the mutational profile and subclone evolution during disease progression can be obtained by the analysis of multiple serial samples.

This is the first study to investigate the mutational status of a large group of MDS patients showing disease progression by the study of serial samples using a NGS myeloid gene panel. We have determined the frequency and chronology of myeloid gene mutation acquisition during disease progression in MDS, identifying specific mutations that are associated with disease evolution, and illuminating the role of subclone development in MDS progression. These data suggest that there are several genetic paths for MDS progression.

CONFLICT OF INTEREST
The authors declare no conflict of interest.
The leukemia-association RUNX1/ETO oncoprotein confers a mutator phenotype

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The leukemia-associated RUNX1/ETO oncoprotein confers a mutator phenotype

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RUNX1/ETO-positive clones revealed that expression of the fusion was stable over long culture periods (>12 weeks). Independent vector control clones that expressed equivalent levels of EGFP to RUNX1/ETO-positive cells were used as fusion-protein negative controls (Supplementary Figure 1D).

PIGA encodes a protein essential for production of the glycosylphosphatidylinositol anchor, mediating anchoring of proteins including CD55 and CD59 to the cell membrane.12 Somatic mutations of PIGA are growth neutral and can be determined using flow cytometry analysis measuring CD55 and CD59 expression (Supplementary Figure 2; Supplementary Methods). Using this reporter gene system we determined the mutation frequency (Mf) in several independent RUNX1/ETO-expressing and vector control clones after continuous culture for 8–10 weeks after initial cloning. All clones had a majority population that was positive for CD55 and CD59, confirming that the founding cell was PIGA wild-type (WT). The mean PIGA Mf in RUNX1/ETO clones (7.50 × 10−4) was five times higher than in vector control clones (1.45 × 10−4) (Figure 1a, Supplementary Figure 3A) (P = 0.032), with the exception of a single clone that had low expression of the fusion transcript (equivalent to 40% of that in Kasumi-1) (Supplementary Figures 1B and 3A).

The frequency of cells with a mutation (Mf) at a specific gene is determined by the mutation rate per cell division for that gene (μ) and the number of cell divisions (d). Expression of the RUNX1/ETO fusion had a modest but nonsignificant negative effect on cell proliferation (Figure 1b, Supplementary Figure 3B, P = 0.812), confirming that the increased Mf in RUNX1/ETO fusion-positive cells was not due to elevated proliferation.

Despite being derived from single cells, EGFP expression in RUNX1/ETO clones was normally distributed in all cell populations (Figure 1c, Supplementary Figure 4), indicating a natural drift in EGFP post cloning. Our data suggest that RUNX1/ETO confers a

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