MYELOID NEOPLASIA

Clinical and biological implications of driver mutations in myelodysplastic syndromes

Elli Papaemmanuil,1 Moritz Gerstung,1 Luca Malcovati,2 Sudhir Tauro,3 Gunes Gundem,1 Peter Van Loo,1,4,5 Chris J. Yoon,1 Peter Ellis,1 David C. Wedge,1 Andrea Pellagatti,6 Adam Shlien,1 Michael John Groves,3 Simon A. Forbes,1 Keiran Raine,1 Jon Hinton,1 Laura J. Mudie,1 Stuart McLaren,1 Claire Hardy,1 Calli Latimer,1 Matteo G. Della Porta,2 Sarah O’Meara,1Ilaria Ambaglio,2 Anna Galli,2 Adam P. Butler,1 Gunilla Walldin,7 Jon W. Teague,7 Lynn Quek,8 Alex Sternberg,6,9 Carlo Gambacorti-Passerini,10 Nicholas C. P. Cross,11 Anthony R. Green,12,13 Jacqueline Boulwood,6 Paresh Vyas,7 Eva Hellstrom-Lindberg,17 David Bowen,14 Mario Cazzola,2 Michael R. Stratton,1 and Peter J. Campbell1,12,13 on behalf of the Chronic Myeloid Disorders working group of the International Cancer Genome Consortium

1Cancer Genome Project, Wellcome Trust Sanger Institute, Hinxton, United Kingdom; 2Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico, Policlinico San Matteo, University of Pavia, Pavia, Italy; 3Division of Medical Sciences, University of Dundee, Dundee, United Kingdom; 4Center for the Biology of Disease, Vlaams Instituut voor Biotechnologie, Leuven, Belgium; 5Department of Human Genetics, Katholieke Universiteit Leuven, Leuven, Belgium; 6Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Oxford, United Kingdom; 7Department of Haematology, Karolinska Institute, Stockholm, Sweden; 8Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom; 9Department of Haematology, Great Western Hospital, Swindon, United Kingdom; 10Department of Haematology, University of Milan Bicocca, Milan, Italy; 11Faculty of Medicine, University of Southampton, Southampton, United Kingdom; 12Department of Haematology, University of Cambridge, Cambridge, United Kingdom; 13Department of Haematology, Addenbrooke’s Hospital, Cambridge, United Kingdom; and 14St. James Institute of Oncology, St. James Hospital, Leeds, United Kingdom

Key Points

- MDS is characterized by mutations in >40 genes, a complex structure of gene-gene interactions and extensive subclonal diversification.
- The total number of oncogenic mutations and early detection of subclonal mutations are significant prognostic variables in MDS.

Myelodysplastic syndromes (MDS) are a heterogeneous group of chronic hematological malignancies characterized by dysplasia, ineffective hematopoiesis and a variable risk of progression to acute myeloid leukemia. Sequencing of MDS genomes has identified mutations in genes implicated in RNA splicing, DNA modification, chromatin regulation, and cell signaling. We sequenced 111 genes across 738 patients with MDS or closely related neoplasms (including chronic myelomonocytic leukemia and MDS–myeloproliferative neoplasms) to explore the role of acquired mutations in MDS biology and clinical phenotype. Seventy-eight percent of patients had 1 or more oncogenic mutations. We identify complex patterns of pairwise association between genes, indicative of epistatic interactions involving components of the spliceosome machinery and epigenetic modifiers. Coupled with inferences on subclonal mutations, these data suggest a hypothesis of genetic “predestination,” in which early driver mutations, typically affecting genes involved in RNA splicing, dictate future trajectories of disease evolution with distinct clinical phenotypes. Driver mutations had equivalent prognostic significance, whether clonal or subclonal, and leukemia-free survival deteriorated steadily as numbers of driver mutations increased. Thus, analysis of oncogenic mutations in large, well-characterized cohorts of patients illustrates the interconnections between the cancer genome and disease biology, with considerable potential for clinical application. (Blood. 2013;122(22):3616-3627)

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Setting to personalize clinical care. The considerable complexity of the clinical phenotype, and whether they can be used in a diagnostic setting, is an increasing focus of complex genetic interactions that de ...

Introduction

Large-scale sequencing of cancer genomes has now been completed for thousands of cancer samples. This initial discovery phase has uncovered many novel genes, pathways, and mutational processes implicated in cancer development. Now, attention is increasingly turning to understanding how these cancer genes knit together, how they influence disease evolution, how they dictate clinical phenotype, and whether they can be used in a diagnostic setting to personalize clinical care. The considerable complexity observed in cancer genomes suggests that such aspirations will only be achieved through comprehensive analysis of large cohorts of well-characterized patients. Although initiation of prospective sample ascertainment is underway, there is considerable potential to address at least in part some of these questions with established cohorts.

Myelodysplastic syndromes (MDS) are hematological malignancies that present with abnormal blood counts and a risk of progression to acute myeloid leukemia (AML). Diagnosis depends on findings in peripheral blood and bone marrow examination, which can show poor interobserver reliability. An increasing number of cancer genes have been found to carry recurrent somatic mutations in MDS, including genes involved in signal transduction (JAK2, KRAS, CBL); DNA methylation (DNMT3A, TET2, IDH1/2); transcriptional regulation (EVII, RUNXI, GATA2); chromatin modification (EZH2, ASXL1); and most recently, RNA splicing (SF3B1, U2AF1, SRSF2 and ZRSR2). Among these mutations, many are shared across the spectrum of myeloid neoplasms (myeloproliferative neoplasms [MPN], MDS/MPN, chronic myelomonocytic leukemia [CMML], and AML) and are likely to dictate morphological and clinical phenotypes.

To explore the interlocking genomic, biological, and clinical features of MDS, we performed a focused screen of 111 cancer genes in a large cohort of MDS patients and closely related neoplasms. Contrary to gene discovery studies that routinely screen matched tumor and constitutional DNA, large-scale gene resequencing is applied to tumor samples only. We developed new computational pipelines for analysis, variant detection, determination of clonal phylogenies from limited number of mutations, and evaluation of combined prognostic accuracy of mutations in >100 genes. This unravels a network of complex genetic interactions that define critical steps in disease progression and identify potential diagnostic and prognostic biomarkers.

Methods

Patient samples and targeted DNA sequencing

Samples were obtained with written informed consent in accordance with the Declaration of Helsinki and appropriate Ethics Committee approvals from 738 patients (Table 1). Of these, 603 had MDS as subclassified by the World Health Organization in 2008 (with the exception of refractory cytopenia with multilineage dysplasia and ringed sideroblasts [RCMD-RS], which we maintain from the World Health Organization, 2002, as a separate category), 70 had CMMML, 35 had progressive disease (MDS-AML), and 13 were of undefined MDS category or classified as MDS-MPN (including refractory anemia with ringed sideroblasts associated with marked thrombocytosis [RARS−T]). Where disease-modifying treatment was administered, duration of follow-up was considered complete without reaching the end-point (“censored”) at the time of starting disease-modifying treatment (specifically, allogeneic stem cell transplantation, aggressive chemotherapy, or hypomethylating agents). Genomic DNA was obtained from peripheral blood granulocytes (n = 431) or bone marrow mononuclear cells (n = 307). Germine DNA was not generally available.

Genomic DNA samples underwent whole-genome amplification. RNA baits were designed to capture a panel of 111 genes (supplemental Table 1, found on the Blood Web site) selected on the basis of prior implication in the pathogenesis of myeloid disease by recurrent somatic mutation; recurrent mutation or aberrations in common cancers; candidates genes from in-house data; or candidate genes mapping within regions of common copy number alterations. Sequencing libraries were generated 96-well format, each carrying a unique DNA barcode (supplemental Figure 1) and sequenced on 2 lanes of an Illumina HiSeq.

To identify base substitutions and small insertions or deletions, we analyzed each sample using in-house algorithms but against an unrelated reference sample. In the absence of a matched control sample, it is challenging to distinguish with perfect accuracy between somatic and germline variants. However, the landscape of truly somatic mutations in these cancer genes has been well established from large-scale genomics studies, allowing confident predictions to be made. To account for the absence of matched control, we developed a bespoke variant selection pipeline applying stringent criteria (see supplemental Methods).

Potential caveats of our protocol could be (1) that whole genome amplification may result in nonuniform representation of the mutations in the diagnostic sample; (2) that artifacts may be introduced during the amplification; or (3) that the proportion of DNA molecules representing a variant may not be reflective of the true allele burden in the diagnostic sample. To test these caveats, we used 6 control datasets: (1) exome sequencing of genomic and constitutional DNA for 10 samples that underwent whole
Table 1. Baseline characteristics of patients in the study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline distribution in cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample</strong></td>
<td></td>
</tr>
<tr>
<td>Sample size for sequencing</td>
<td>738</td>
</tr>
<tr>
<td>Sample size with outcome data</td>
<td>595</td>
</tr>
<tr>
<td>Median (range) follow-up</td>
<td>12 (0-155) months</td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>415 (56%)</td>
</tr>
<tr>
<td>Female</td>
<td>323 (44%)</td>
</tr>
<tr>
<td>Age, mean ± SD</td>
<td>68 ± 13 years</td>
</tr>
<tr>
<td><strong>MDS classification</strong></td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>139 (19%)</td>
</tr>
<tr>
<td>RARS</td>
<td>92 (12%)</td>
</tr>
<tr>
<td>RARS-T</td>
<td>17 (2%)</td>
</tr>
<tr>
<td>RCMD</td>
<td>126 (17%)</td>
</tr>
<tr>
<td>RCMD-RS</td>
<td>59 (8%)</td>
</tr>
<tr>
<td>RAEB</td>
<td>167 (23%)</td>
</tr>
<tr>
<td>5a</td>
<td>20 (3%)</td>
</tr>
<tr>
<td>CMML</td>
<td>70 (9%)</td>
</tr>
<tr>
<td>MDS-MPN</td>
<td>3 (0.4%)</td>
</tr>
<tr>
<td>MDS-U</td>
<td>10 (1%)</td>
</tr>
<tr>
<td>MDS-AML</td>
<td>35 (5%)</td>
</tr>
<tr>
<td><strong>Blood counts at diagnosis</strong></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin level, mean ± SD</td>
<td>9.9 ± 2.0 g/dL</td>
</tr>
<tr>
<td>Neutrophil count, mean ± SD</td>
<td>3.0 ± 3.4 x 10^9 per liter</td>
</tr>
<tr>
<td>Platelet count, mean ± SD</td>
<td>191 ± 161 x 10^9 per liter</td>
</tr>
<tr>
<td><strong>Bone marrow features</strong></td>
<td></td>
</tr>
<tr>
<td>Ring sideroblasts &gt; 15%</td>
<td>173/529 (33%); missing for 209 patients</td>
</tr>
<tr>
<td>Bone marrow blasts &gt; 5%</td>
<td>202 (27%)</td>
</tr>
<tr>
<td><strong>Karyotype</strong></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>425</td>
</tr>
<tr>
<td>Abnormal</td>
<td>213</td>
</tr>
<tr>
<td>Failed/not done</td>
<td>100</td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td></td>
</tr>
<tr>
<td>Leukemic transformation</td>
<td>66/595 (11%)</td>
</tr>
<tr>
<td>Died</td>
<td>234/595 (39%)</td>
</tr>
</tbody>
</table>

MDS-U, undefined MDS category; RAEB, refractory anemia with excess blasts; SD, standard deviation.

### Statistical analysis

Pairwise associations between genes were evaluated by Fisher tests corrected for multiple hypothesis testing. For the 595 patients with available outcome data, leukemia-free survival was the end-point, and log-rank tests were used for univariate hypothesis tests. For multivariate survival analyses, missing data were estimated by multiple imputation, and Cox proportional hazards models were built from 3 sets of predictor variables using stability selection. Accuracy of outcome predictions was averaged across 5 cross-validation samples from models built on the remaining 4 out of 5 patients. We use the least absolute shrinkage and selection operator variable selection in Cox’s proportional hazards method and receiver operating characteristic curves to illustrate predictive accuracy.

### Results

#### Targeted gene sequencing in MDS

We sequenced 111 genes across 738 patients, resulting in 2260 high-confidence variants (supplemental Table 2). Coverage across the targeted regions was excellent (supplemental Figure 2).

The aim was to define for each patient the potential driver mutations implicated in their disease. In keeping with widely accepted conventions in the genomics literature, we used a pragmatic, purely genetic definition of driver mutations, defined on the basis of published studies describing a statistically significant excess of somatic mutations in a given cancer gene. The expected pattern of somatic mutations in the given gene was defined from the literature, typically inactivating mutations for tumor suppressor genes and hot-spot mutations for oncogenes. Every variant identified in this study was then compared against these expected patterns and triaged into “driver mutations,” “possible oncogenic variants,” or “unknown significance” (see supplemental Methods for further details). This definition of driver mutation is not dependent on whether there is functional evidence of oncogenic potential.

#### Study controls

To assess whether absence of germline DNA would annotate rare germline polymorphisms as driver mutations, we evaluated calls identified by gene resequencing to those identified by exome analysis from matched tumor and constitutional samples in 10 patients. Of 21 somatic variants found in the 111 genes in the current panel, all were identified, and 20 out of 21 (supplemental Table 3) passed the stringent filtering criteria applied (95.2% sensitivity). We analyzed the exomes through our unmatched pipeline; all mutations annotated as drivers were indeed somatic in the exome data. No oncogenic variants were called in any of the 78 (22 + 56) constitutional samples.

We tested sensitivity and specificity of the protocol in comparison with orthogonal sequencing approaches. Of 147 known SF3B1 mutations in the cohort, all were identified, and we called an additional 11 missed originally because of poor coverage. Similarly, for the cohort of 184 patients with known TET2 status, we recaptured 20 out of 21 mutations (95%) and called an additional 3.

To test whether WGA biased allele representation, we analyzed sequencing data from a subset of patients in whom native and amplified DNA had been studied. Variant allele fractions from WGA samples were not significantly different from those from the same patients’ genomic DNA (supplemental Tables 3-4), consistent with published findings. The overall distribution of variant allele fractions for variants classified as oncogenic or possible oncogenic had the same distribution as reported for validated mutations identified by exome sequencing (supplemental Figure 3A-B) and was clearly distinct from that for variants known to be germline polymorphisms (supplemental Figure 3C).

These control data show that our design does not lead to significant over- or undercalling of driver mutations, systematic biases in allele fraction estimates, or excessive numbers of germline variants miscalled as driver mutations.
Figure 1. Genomic architecture of MDS. (A) Frequency of driver mutations identified in the sequencing screen or by cytogenetics in the cohort of 738 patients, broken down by MDS subtype. (B) Example of a copy number plot from a patient with a cytogenetically proven deletion on chromosome 5q. The upper panel depicts the normalized sequencing yields per exon; the lower panel depicts the variant allele fraction for germline SNPs. "AB" indicates the expected B-allele fractions for heterozygous SNPs; "AA" and "BB" indicate the position of the expected B-allele fractions for the homozygous SNPs AA and BB. (C) Associations among genes and cytogenetic abnormalities with disease subtypes in the study. Only associations with a q value (P value corrected for multiple hypothesis testing) $<$ 0.1 are shown. Associations are colored by odds ratio. Blue-green colors depict gene-subtype associations that are observed together more than expected by chance, with brown colors depicting gene-subtype associations observed together less frequently than expected by chance.
Gene mutations in MDS and related neoplasms

Oncogenic mutations were identified in 43 genes (Figure 1A). The splicing factor SF3B1 was the most frequently mutated in the cohort (24%), followed by TET2 (22%) and SRSF2 (14%). Only 4 genes were mutated in more than 10% of patients, with a further 3 genes carrying driver mutations in 5% to 10% patients. Notably, 36 genes were mutated in <5% of the patients, and in aggregate, mutations in these genes contribute 33.5% of all mutations identified. Among these, we found oncogenic mutations in IRF1, which we previously identified in 1 patient with RARS\textsuperscript{11} as well as the recently reported gene in AML, CUX1.\textsuperscript{31} Mutations in well-known cancer genes not previously implicated in MDS (EP300, CREBBP, and PTEN) were also observed. These variants are rare (<2%) but follow the same distribution of nonsense, splice, and frameshift mutations as seen in other cancers.

Detection of copy number changes from sequencing data

With cytogenetic abnormalities found in up to 40% of MDS patients, we assessed whether counts of sequencing reads could distinguish copy number aberrations (Figure 1B). Of 738 patients sequenced, credible copy number profiles were generated from 629 (85%). Abnormalities were seen in 101 (13%) patients, including deletions of 5q, 11q, 20q, and 17p; monosomy 7; trisomies of 8 and 21; and isochromosome X (supplemental Figure 5, supplemental Table 5). Importantly, in addition to common copy number alterations, lesions
invisible to cytogenetics, such as uniparental disomy, were identified (supplemental Figure 5). Our findings suggest that with further optimization of this preliminary design, potentially by targeting germline single nucleotide polymorphism (SNPs), the sensitivity to detect clinically relevant copy number alterations could be increased. This would enable the simultaneous detection of both gene mutations and cytogenetic abnormalities in a single assay but requires further evaluation.

Oncogenic mutations identified in 78% patients with MDS

In total, 549 of 738 (74%; 95% CI, 71% to 77%) patients had at least 1 oncogenic point mutation or MDS-related copy number change detectable by sequencing (Figure 2A), whereas cytogenetic studies identified abnormalities in 33%. When sequencing and cytogenetics were combined, the fraction of patients with MDS-related oncogenic lesions increased to 78%. Indeed, 43% patients had 2 or more driver mutations, and 10% had 4 to 8 (Figure 2B).

We searched for pairwise gene associations, recognizing that pairs of genes could show a tendency to either cooccurrence or mutually exclusivity. Forty-six pairs were significant with false discovery rate, 10% (Figure 2C; supplemental Table 6). Several of these have been reported previously.5,10,34-36 Mutually exclusive gene pairs often imply functional redundancy, especially if such genes are in the same biological pathway. Indeed, mutations in genes involved in the RNA splicing machinery were mutually exclusive, as in other studies.10,35 This implies that any one of these mutations is sufficient by itself, with no additional advantage accruing from more than 1 mutation in this pathway. Similarly, we confirm previous studies showing mutual
Figure 4. Outcome by whether driver mutations are clonal or subclonal. Leukemia-free survival for patients showing no mutation (gray), clonal driver mutations (blue), or subclonal driver mutations (red) for (A) TET2, (B) ASXL1, (C) SRSF2, (D) EZH2, (E) CBL, and (F) RUNX1. The P values denote the hypothesis test of whether splitting driver mutations into clonal or subclonal categories improves fit in a Cox proportional hazards model.
exclusivity between mutations in TET2 and IDH2, both linked to
disordered DNA hydroxymethylation.34

Functional redundancy, however, does not explain all the ob-
served mutually exclusive associations. For example, EZH2 and
SRSF2 mutations were never found together (q = 0.04), although
they seemingly operate in different pathways. Similarly, we observe
mutually exclusive associations between some genes and cyto-
genetetic lesions as well as between IDH2 and SF3B1 (Figure 2C).
In the latter case, it is striking that IDH2 shows a clear proclivi-
ity for combination with SRSF2 (odds ratio, 6.7; 95% CI, 4.9–9.3;
q = 0.0004), whereas SRSF2 is mutually exclusive with SF3B1.
Thus, apart from functional redundancy, another explanation for
mutually exclusivity is that some genes may only be transforming in
specific genomic contexts.

In fact, SF3B1 and SRSF2 show striking differences in their sets
of comutated genes. Thus, despite both genes being involved in the
same pathway, the sets of comutated genes are different, implying
that the functional consequences on RNA splicing cannot be iden-
tical. Furthermore, the fact that SF3B1 is linked to myelodysplasia
with ring sideroblasts,10,11,37 whereas SRSF2 is particularly en-
riched in CMML30 (Figure 1C), indicates that major differences
in disease phenotype can be driven by different combinations of
comutated genes. Such relationships appear to underlie patterns
of comutation in the study (supplemental Figure 6). These net-
works of interacting genes provide important clues to the biology
of MDS. For example, a mouse model combining Axcfl loss and
Nras, which are comutated, showed a more aggressive, penetrant
disease than did either lesion alone,38 confirming a biologically
relevant interaction.

Clonal architecture in MDS describes preferred trajectories of
disease evolution

During cancer development, functional mutations drive sequential
waves of clonal expansion, and parallel sequencing has enabled this
process to be characterized in some detail.39–41 Clonal evolution has
been documented as MDS transforms to AML,42 and when de novo
AML relapses after chemotherapy.43 Variant allele fractions can be
used to estimate the proportion of tumor cells carrying a given
mutation and identify clonal mutations (in all cells) or subclonal
(mutations, 62% showed only clonal driver mutations, and in a
further 4% the subclonal fractions were too low to reconstruct phy-
logenetic relationships (Figure 3B). The remaining 34% of patients
had strong statistical evidence for the existence of clonal as well as
subclonal driver mutations in which we could robustly define a set
of pairwise precedences reflecting the temporal order of acquisition.
With the large sample size of patients available, there were clear
trends across the set of mutated genes, with some occurring con-
sistently earlier than others (supplemental Figure 7).

Using these pairwise precedences, we calculated a global ranking
of MDS genes reflecting how early in disease evolution they are
mutated (Figure 3C). Strikingly, mutations in genes involved in RNA
splicing and DNA methylation occur early, whereas driver mutations
in genes involved in chromatin modification and signaling often occur
later. These are not absolute rules (supplemental Figure 7) but
establish robust trends for the observed temporal acquisition.

Our data suggest a hypothesis of genetic “predestination,” that
eyearly mutations shape the future trajectories of clonal evolution of a
cancer through constraints on the repertoire of cooperating genetic
lesions. Here, we find that splicing factors such as SF3B1 and SRSF2
are typically mutated early. However, the 2 genes exhibit pronounced
and contrasting preferences for which genes are most likely to pro-
vide selective advantage subsequently, driving considerable mor-
phologic differences (Figure 1B). We note that this hypothesis is
based on inferences from a cross-sectional study. Confirmation
would require longitudinal analyses of serial samples drawn from
large cohorts.
Clonal and subclonal mutations affect prognosis equally

Follow-up data were available for 595 patients (Table 1). Of 24 genes mutated in >5 patients, 8 genes were associated with significantly worse leukemia-free survival if mutated and 1 gene (SF3B1) with a better leukemia-free survival (supplemental Figure 8). These findings replicate previous studies.5,9,11,35-37,45,46

We assessed whether the effects on clinical outcome of a given gene differed by whether mutations were clonal or subclonal. To explore this, we compared leukemia-free survival of patients with mutations in the dominant clone to that of patients with mutations in the same gene present in a minor subclone. Strikingly, we found no significant difference in leukemia-free survival between clonal and subclonal mutations for the 6 genes with published survival effects11,35,46 in which we observed at least 5 patients with subclonal driver mutations (Figure 4), highlighting the importance of detecting these subclonal mutations. With a challenging classification and a chronic clinical course, this information could enable early identification of high-risk patients as well as the detection of new emerging subclones of prognostic significance.

Outcome correlates with number of driver mutations

In our study, leukemia-free survival negatively correlates with the combined number of oncogenic mutations and cytogenetic lesions \((P < .0001; \text{Figure 5A})\). This remains true if only oncogenic gene mutations (excluding cytogenetic aberrations) are considered \((P = .002, \text{supplemental Figure 9})\) and remains significant independent of TP53 or SF3B1 mutation status. The estimated median leukemia-free survival for patients with 1 oncogenic mutation or cytogenetic lesion was 49 months, dropping to 42, 27, 18, and 4 months for patients with 2, 3, 4 to 5, and >6 mutations, respectively. This was mirrored by a monotonic increase in rates of transformation to acute leukemia as the number of driver variants increased \((P < .0001; \text{Figure 5B})\). These data chime with observations that transformation from MDS to AML or relapse of de novo AML is
driven by clonal evolution associated with acquisition of new driver mutations.\textsuperscript{32,43}

The International Prognostic Scoring System (IPSS), revised in 2012,\textsuperscript{33} is the most widely used prognostication scheme in MDS. Gene mutations are currently not included, although there are data to show that prognostic prediction can be improved by their inclusion.\textsuperscript{35} We find that the number of oncogenic mutations continues to provide independent prognostic information after stratification by the IPSS classification (\(P = .0004\); supplemental Figure 9).

Twenty-two percent of MDS patients showed no evidence of known oncogenic point mutations or cytogenetic aberrations. Patients with no identified oncogenic events show leukemia-free survival and rates of transformation to acute leukemia similar to those with 1 to 2 driver mutations (Figure 5A-B). This suggests that they have a disease course typical of MDS. Several possible explanations may underlie why we did not identify any mutations in this group. Because we identified no systematic differences in the overall distribution of driver mutations between samples derived from bone marrow and those from peripheral blood, source of DNA is not a major factor. There are several new genes that are targets for recurrent mutation in myeloid malignancies—such as \textit{SETBP1}, \textit{SMC1A}, and \textit{SMC3}—\textsuperscript{37,48}—that were published after this study was performed and could account for a proportion of the unaccounted patients.

Furthermore, even in well-characterized genes, there could be rare driver mutations. For example, we found 10 variants in \textit{ASXL1} at residues not previously characterized and consequently annotated as variants of unknown significance. The prognosis for the 10 patients with these mutations was significantly worse than for patients without \textit{ASXL1} variants (\(P = .03\), log-rank test) and tracked the survival curve for patients with known oncogenic mutations (Figure 5C). This suggests that at least some of these variants may be of functional importance, although definitive proof would require establishing that they are somatically acquired, recurrent in a larger cohort, and had prognostic effects independent of other variables. Albeit exploratory, this analysis suggests that larger sample sizes with matched-to-clinical data will support identification of rare driver mutations.

Making prognostic predictions from sequencing data

The refinement of the composite genetic architecture that underpins MDS has led to a growing anticipation of how these findings can be translated into clinical practice. We therefore explored what proportion of the variance in clinical outcomes could be accounted for by clinical and genomic features. These include morphological variables, demographic data, peripheral blood counts at diagnosis, cytogenetics, and gene mutations.

We considered 3 potential datasets: the IPSS; a dataset derived from all standard clinical variables (including peripheral blood counts, bone marrow morphology, cytogenetics, and demographic data); and a dataset that combines standard and genetic variables together. Owing to missing data, we were not able to calculate IPSS—Revised (IPSS-R) status. For each dataset, the variables that had sufficient independent predictive power to enter these models are detailed in supplemental Table 7. When compared with the IPSS (area under the curve [AUC] = 0.76 at 90 months), standard variable sets show an increase in the prognostic potential (AUC = 0.80 at 90 months) in relation to that obtained by the IPSS alone (Figure 6A). This is in accordance with recent observations from the IPSS-R, which has refined the incorporation of further cytogenetic abnormalities as well as the degree of cytopenias and bone marrow blast percentage.\textsuperscript{33} Incorporation of the point mutation data achieves a marginal nonsignificant increase (AUC = 0.82 at 90 months), but the 2 curves are broadly overlapping. This indicates that the amount of prognostic information contained in each of the 2 datasets is similar and implies that there is some redundancy in the prognostic information between these 2 sets.

We have previously shown that the \textit{SF3B1} mutation status is a significant predictor for the presence of ringed sideroblasts in the bone marrow.\textsuperscript{37} To evaluate other such genotype-phenotype correlations, we used multivariate models to predict clinical variables of prognostic significance (such as ringed sideroblasts, hemoglobin count, bone marrow blasts) using driver mutations (point mutations and cytogenetic alterations) as the predictors (Figure 6B-C). \textit{TET2} mutations and del(5q) were the most important genetic predictors of hemoglobin levels, the former being positively correlated and the latter negatively correlated (Figure 6B). In combination, genetic variables can explain 0.063% of the variance observed in hemoglobin levels. Similarly, mutations in \textit{WT1}, \textit{IDH2}, \textit{STAG2}, and \textit{NRAS}, as well as a complex karyotype, correlated strongly with percentage of bone marrow blasts, whereas \textit{SF3B1} mutations predicted a low fraction of blasts (Figure 6C).

Taken together, these data demonstrate that inclusion of genomic data should improve prognostic algorithms for MDS. Given that many genes are rarely mutated and show the complex patterns of comutation, much larger sample sizes will be required to realize this potential. The IPSS-R used 7012 patients\textsuperscript{33}; a similar sample set analyzed with the protocol outlined here may be necessary for robust prognostic schemes that incorporate genomic variables.

Discussion

The large sample size and extent of gene sequencing reported here provides an unprecedented glimpse into the genomic landscape of MDS and how this impacts on clinical phenotype. For the first time, we have performed targeted gene resequencing of a clinical cohort in the absence of constitutional matched DNA. We have developed several computational approaches to deal with data sets on this scale, especially in measuring combined prognostic information and inferring temporal evolution of gene mutations. We observe the same frequencies of mutations in specific genes as reported in the literature, confirm known gene-gene interactions, and validate published correlations with patient outcome.

We identified at least 1 genomic alteration in 78% of the patients studied. In the time since our bait set was synthesized, several new myeloid genes have been described, including \textit{SMC1A}, \textit{SMC3}, and \textit{SETBP1};\textsuperscript{37,48} meaning that this figure could be improved in the future. A conservative variant annotation was used, with many variants classified as of “unknown significance”; larger sample sizes may enable some of these to be reclassified in the future.

In MDS, several key observations emerge. Many genes are targets for mutation in MDS, but the vast majority are rare (<5%). One of the strongest predictors of outcome is the number of driver mutations identified in a patient. Twenty percent of mutations in patients with 1 driver mutation map to genes mutated in <2% of cases. This is also true for 30% to 40% of additional acquired mutations (3rd, 4th, etc.). Thus, for use in diagnostic screening, sequencing a comprehensive set of well-characterized genes is critical.

RNA splicing is the most commonly mutated pathway in MDS, and we find strong evidence that mutations in splicing factors occur early in disease evolution. These mutations play a major role in determining the clinical features of the disease, with differences in morphological features seen on bone marrow biopsy and in
leukemia-free survival. Intriguingly, not only do these mutations occur early, but they may also influence the subsequent genomic evolution of the disease, because the patterns of cooperating mutations are strikingly different between, for example, SF3B1 and SRSF2. Confirmation of this hypothesis would require analysis of serial samples.

It will be increasingly feasible to undertake sequencing of DNA from sequential blood samples on, say, an annual basis in MDS patients. Our data suggest that the emergence of new driver mutations, even if they are still subclonal, can have significant implications for the future disease course. It should therefore be possible to identify patients whose disease is progressing before symptoms associated with higher-risk disease are manifested.

There has been considerable excitement about the opportunity that massively parallel sequencing offers as a cost-effective, frontline diagnostic tool for cancer. Our study is a harbinger of future comprehensive genomic analyses of large cohorts of patients with clinical data across different tumor types. Many of the themes seen here will emerge repeatedly, providing important insights into the genomic architecture of cancer and how this drives the phenotypic and clinical heterogeneity we see in patients.

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Authorship


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Correspondence: Peter Campbell, Wellcome Trust Sanger Institute, Hinxton, CB10 1SA, United Kingdom; e-mail: pc8@sanger.ac.uk.

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Clinical and biological implications of driver mutations in myelodysplastic syndromes

Elli Papaemmanuil, Moritz Gerstung, Luca Malcovati, Sudhir Tauro, Gunes Gundem, Peter Van Loo, Chris J. Yoon, Peter Ellis, David C. Wedge, Andrea Pellagatti, Adam Shlien, Michael John Groves, Simon A. Forbes, Keiran Raine, Jon Hinton, Laura J. Mudie, Stuart McLaren, Claire Hardy, Calli Latimer, Matteo G. Della Porta, Sarah O’Meara, Ilaria Ambaglio, Anna Galli, Adam P. Butler, Gunilla Walldin, Jon W. Teague, Lynn Quek, Alex Sternberg, Carlo Gambacorti-Passerini, Nicholas C. P. Cross, Anthony R. Green, Jacqueline Boulwood, Paresh Vyas, Eva Hellstrom-Lindberg, David Bowen, Mario Cazzola, Michael R. Stratton and Peter J. Campbell

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