Targeted Sequencing Informs the Evaluation of Normal Karyotype Cytopenic Patients for Low-Grade Myelodysplastic Syndrome

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The diagnosis of myelodysplastic syndrome (MDS) requires persistent cytopenias, not otherwise explained, and evidence of morphologic dysplasia in the bone marrow. Low-grade MDS (bone marrow blasts <5%) has morphologic dysplasia in at least 10% of cells in one or more cell lineages.¹ Low-grade MDS is particularly challenging to diagnose, as no definitive criteria for morphologic dysplasia exist and evaluation may be subject to high inter-observer variability.¹⁻³ The ability to diagnose low-grade MDS can be improved by incorporating cytogenetic evaluation of the bone marrow, especially in the setting of equivocal morphologic dysplasia. However, many MDS cases (up to 60%) lack cytogenetic abnormalities, limiting the overall utility of cytogenetics as a diagnostic adjunct.⁴

Multiple studies have demonstrated that the majority of MDS patients (~80% in some studies) harbor recurrent somatic mutations in a group of 20-30 genes.⁵⁻⁷ Further, some gene mutations confer an adverse prognosis independent of clinical scoring systems.⁵, ⁶, ⁸ We sought to determine whether targeted DNA sequencing of recurrently mutated MDS genes could be a useful adjunct in the diagnostically challenging subgroup of cytopenic
patients with low blast counts and a normal karyotype, thereby identifying a subset of patients that may potentially be at a higher risk of developing MDS or AML.

We screened 599 patients who presented between 1/2002 and 11/2015, consented for sequencing studies on a protocol approved by the Human Research Protection Office at Washington University, and had banked bone marrow and control tissue (skin). Forty-three patients were selected based on 1) stringent cytopenia criteria (WBC <1,800/μL, hemoglobin <10g/dL, platelets <100k/μL) in at least one lineage, 2) bone marrow blasts <5% by flow cytometry and/or morphologic evaluation (and had slides available for review) 3) WBC <14k/μL, 4) non-clonal metaphase cytogenetics, and 5) absence of prior therapy for MDS (Table 1). Bone marrow specimens were independently reviewed (blinded) for blast count and dysplasia by two board-certified hematopathologists (ED and KV) and the percentage of dysplastic cells in the myeloid, erythroid, and megakaryocytic lineages enumerated. Dysplasia was binned into categories of <10%, 10-20%, 21-50%, and >50%. Definitive dysplasia was established when both pathologists identified dysplasia in ≥10% of cells in at least one lineage. Equivocal dysplasia was rendered when there was disagreement over the identification of ≥10% dysplasia in at least one lineage. No dysplasia was rendered when both pathologists agreed that dysplasia was <10% in all lineages. Genomic DNA was extracted from bone marrow and skin (as a source of normal DNA) and enriched for the coding exons of a panel of 284 commonly mutated myeloid genes (Supplementary Table 1). DNA was extracted from aspirate coverslips for follow-up cases when cryopreserved cells were not available. Libraries were sequenced on a HiSeq 2500 (Illumina, San Diego, CA) instrument with 2×101 bp reads. The resulting data was analyzed for single nucleotide variants (SNVs) and insertions/deletions (indels), using standard analysis pipelines in paired normal mode, as previously reported. To reduce false positive calls, only variants with ≥5 variant reads, ≥50x total coverage in marrow and skin samples, ≥5% variant allele fraction (VAF, variant reads/total reads) in the marrow, not present in dbSNP (unless known canonical somatic hotspot mutations), and that resulted in protein coding changes were conservatively included in the analysis. Copy number alterations (CNAs) and loss of heterozygosity were called using the CopyCAT2 package.

Mean unique coverage depth was 265x for primary bone marrows, 252x for skin, and 388x for follow-up coverslips. Of the 43 sequenced cases, 29 had a coding-region somatic mutation in at least one gene (mean 2.8 mutations/case, range 1-8 mutations/case). The most commonly mutated gene was SRSF2 (8 cases), followed by TET2 (7 cases), SF3B1 (6 cases), and U2AF1 (6 cases), (Figure 1a). Of the 284 sequenced genes, 40 were mutated in at least one case, and 12 were mutated in 2 or more cases. The mean VAF of SNV mutations was 29.9% (range 5-98%). Co-occurrence data is presented in Supplementary Figure 1.

Morphologic review of bone marrow demonstrated definitive dysplasia (≥10% of cells in at least one lineage) made by two pathologists in 28 cases, establishing the diagnosis of MDS. No significant dysplasia (<10% in any lineage) was seen in 8 cases, and equivocal dysplasia (where hematopathologists did not agree that dysplasia was present in ≥10% cells in at least one lineage) in 7 cases. Twenty-one of 28 cases (75%) with definitive dysplasia (i.e., MDS) and normal cytogenetics had a somatic coding region mutation in at least one gene. Three of 8 cases (37.5%) without dysplasia had mutations and 5 of 7 (71%) cases with equivocal dysplasia had mutations.
dysplasia harbored somatic mutations (Figure 1b). There was no significant difference in mutation VAFs or maximum VAF per patient between the dysplasia and no dysplasia groups (Figure 1c). Cases with dysplasia or equivocal dysplasia had more mutations per case than those without dysplasia (p=0.018 and p=0.036, respectively) (Figure 1d). The fraction of cases with mutations tended to be higher for the dysplasia versus no dysplasia group (p=0.086) (Figure 1b). No copy number altered regions were detected, although UPN609948 showed copy-neutral loss-of-heterozygosity on chromosome 7 (Supplementary Figure 2).

Mutations were detected in 8 patients with equivocal (n=5) or no dysplasia (n=3) and 6 of these 8 patients developed high-grade MDS or had persistent cytopenias requiring pharmacologic treatment. Follow-up data from 5 patients with equivocal dysplasia and somatic mutations showed that 2 developed blast counts >5% (UPN568547, UPN976842) with persistence of mutations and 3 received MDS treatment. UPN701797 had persistent anemia responsive to erythropoietin, UPN724989 was responsive to filgrastim, and UPN728125 had cytopenia improvement following decitabine treatment (Figure 1e). Of the 3 patients with no dysplasia who had somatic mutations, UPN204802 was treated with erythropoietin, UPN859688 subsequently died due to multiple comorbidities without MDS, and UPN529198 had severe iron deficiency anemia secondary to short bowel syndrome (responsive to intravenous iron) and a persistent TET2 mutation without MDS (Figure 1e).

No mutations were detected in 7 patients with equivocal (n=2) or no dysplasia (n=5) and only 2 of these 7 patients were empirically treated as MDS or diagnosed as MDS, and none progressed to high-grade MDS. The 2 patients with equivocal dysplasia and no mutations were diagnosed with hypereosinophilic syndrome (UPN786953) and anemia secondary to end stage renal disease without progression to MDS (UPN610864) (Figure 1e). Of the 5 patients with no dysplasia or somatic mutations, UPN577914 developed MDS with a non-clonal deletion on chromosome 7 after presenting with an autoimmune anemia. No mutations were identified on subsequent sequencing. UPN976020 was diagnosed with an autoimmune cytopenia that fully recovered and UPN163943 had count recovery with erythropoietin treatment. The remaining 2 patients were diagnosed with severe aplastic anemia and treated with an allogeneic bone marrow transplant and cyclosporin (UPN769282, UPN332207, respectively) (Figure 1e).

In this cohort, 5 of 7 (71%) cytopenic patients with blasts <5% and equivocal dysplasia had a somatic mutation in their bone marrow cells, similar to the frequency for cytogenetically normal MDS patients with blasts <5% (21/28, 75%). In contrast, somatic mutations were detected in 3 of 8 cases (37.5%) without definitive dysplasia (Figure 1b). Patients with cytopenias and somatic MDS-associated mutations, but without definitive dysplasia, fit the newly described category of clonal cytopenia of undetermined significance (CCUS). (10) Kwok and colleagues showed that CCUS patients have a similar spectrum of mutated genes and VAFs as patients with bona fide MDS, similar to our findings. (11) Cargo and colleagues recently showed that 91% of ‘pre-diagnostic’ marrows from cytopenic patients who went on to MDS or AML harbored driver gene mutations, suggesting they progressed from an antecedent CCUS. (12) In contrast, the spectrum of mutations in our cohort differs from individuals with clonal hematopoiesis with indeterminate potential (CHIP) - defined by

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mutations but no cytopenias - where 50% of cases have a DNTM3A mutation. In our study, DNTM3A was mutated in <5% of patients and JAK2 and TP53 were not mutated (genes observed in CHIP). The persistent TET2 mutation in UPN529198 may represent a CHIP mutation.

In contrast to prior work by Cargo et al and Kwok et al, this study focused solely on the diagnostically challenging group of patients with cytopenias and normal cytogenetics (i.e., no evidence of clonal disease) and sequenced a larger number of myeloid associated genes using paired normal tissue to definitively call somatic mutations. Similar to Kwok et al, we show that while the mean VAF and maximum VAF is similar between patients with dysplasia and no-dysplasia, patients with dysplasia have an increased number of mutations per case. Further, using follow-up clinical data and subsequent bone marrow biopsies we show that it is more common for cytopenic patients with equivocal/no dysplasia and a gene mutation to be subsequently diagnosed or empirically treated for MDS compared to patients without a mutation (6/8 versus 2/7, respectively). The data suggest that the presence of a gene mutation in a cytopenic patient may be associated with increased risk of developing MDS and provide a rationale for future prospective studies.

Ultimately, sequencing-based evaluation may also provide a means for tracking tumor burden and monitoring patients for subsequent clonal expansion or development of definitive MDS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Gene mutations present in cytopenic patients and subsequent clinical outcome
(a) Distribution of recurrent gene mutations by morphologic category in cytopenic patients. A total of 40 genes were mutated at least once. Colors indicate the morphologic classification: dysplasia (blue), equivocal dysplasia (orange), no dysplasia (green). (b) Frequency of cases in each diagnostic category with at least one somatic mutation. (c) Somatic mutation variant allele fractions (VAFs) by morphologic category. Red points indicate the maximum VAF for each case; blue bars indicate the median VAF for each category. (d) Number of somatic mutations per case for each diagnostic category. There was
a significant difference between the number of mutations per case in the dysplasia vs. no
dysplasia and equivocal dysplasia vs. no dysplasia categories (student’s t-test); blue bars
indicate median values. (e) Follow-up data for patients in the equivocal or no dysplasia
categories grouped by mutational status. The length of the bar indicates the duration of
follow-up; black triangles indicate when patients were treated for MDS; blue triangles
indicate when follow-up sequencing was performed.
### Clinical Characteristics of Study Patients

| Common Morphologic Diagnosis | UPN  | Age  | Sex | WBC  | Hgb  | Pts | Myeloid Dysplasia * | Erythroid Dysplasia * | Megakaryocytic Dysplasia * | Blasts * | Genes with Somatic Mutations | Follow-Up Interval (days) | Treated for MDS |
|------------------------------|------|------|-----|------|------|-----|---------------------|-----------------------|--------------------------|----------|-----------------------------|-------------------------|-----------------|
| Dysplasia                    | 178791 | 72   | M   | 2.9  | 9.5  | 131 | 10-20%<10%         | <10%<10-20%           | 21%-30%                 | 1-3%     | SF3B1, DOCK2                | NA                      | NA              |
| Dysplasia                    | 215065 | 75   | M   | 2.7  | 11.9 | 49  | <8%                | <10%                  | >10%                    | 1-3%/4-5% | IDH2, SRSF2                | NA                      | NA              |
| Dysplasia                    | 233604 | 56   | F   | 5.7  | 12.1 | 17  | <8%                | <10%                  | 10-20%                  | 1-3%     | ASXL1, U2AF1                | NA                      | NA              |
| Dysplasia                    | 357137 | 42   | F   | 2.3  | 9.1  | 127 | <8%                | <10%<10-20%           | 10-20%                  | 1-3%     | CB1, IDH1, SRSF2            | NA                      | NA              |
| Dysplasia                    | 364510 | 47   | M   | 5.2  | 9.7  | 138 | <8%                | <10%/10-20%           | 10-20%<21%-30%        | 1-3%     | None                        | NA                      | NA              |
| Dysplasia                    | 379001 | 80   | F   | 3.7  | 6.9  | 38  | <8%                | <10%                  | >10%                    | 1-3%     | GNB1, U2AF2, U2AF1, FAM47A, ASXL1 | NA                      | NA              |
| Dysplasia                    | 397410 | 82   | M   | 10.3 | 9.5  | 157 | <8%                | <10%                  | 10-20%>50%             | 1-3%     | SRSF2                       | NA                      | NA              |
| Dysplasia                    | 400904 | 71   | F   | 4    | 9    | 43  | <8%                | <10%/10-20%           | 10-20%                  | 1-3%     | ASXL1, DDX1, GATA2          | NA                      | NA              |
| Dysplasia                    | 445082 | 56   | F   | 2    | 10.4 | 82  | <10%<10-20%        | 10-20%<21%-30%        | 1-3%                   | 1-3%     | CB1, IDH1, SRSF2            | NA                      | NA              |
| Dysplasia                    | 469660 | 75   | F   | 3.2  | 9.5  | 38  | <8%                | <10%                  | 10-20%<50%             | 1-3%     | None                        | NA                      | NA              |
| Dysplasia                    | 479751 | 63   | F   | 3.4  | 9    | 340 | <10%<10-20%        | 10-20%<21%-30%        | 1-3%                   | 1-3%     | ASXL1, SETBP1, U2AF1        | NA                      | NA              |
| Dysplasia                    | 501812 | 30   | M   | 4.2  | 8.4  | 92  | <8%                | 10-20%<21-30%         | 21-30%                  | 1-3%     | CB1, IDH1, SRSF2            | NA                      | NA              |
| Dysplasia                    | 567350 | 35   | M   | 3.1  | 12.4 | 70  | <8%                | 10-20%<10%            | <10%/10-20%            | 1-3%     | None                        | NA                      | NA              |
| Dysplasia                    | 583362 | 31   | M   | 3.5  | 11.5 | 83  | <10%/10%<20%       | <10%                  | >10%                    | 1-3%/4-5% | KCN1, IDH1, SRSF2, ASXL1   | NA                      | NA              |
| Dysplasia                    | 584890 | 89   | F   | 2.5  | 8.7  | 196 | 21-50%<10%         | 10-20%<20%            | 1-3%                   | 1-3%     | SF3B1                       | NA                      | NA              |
| Dysplasia                    | 589909 | 36   | F   | 9.9  | 7.8  | 84  | <8%                | <10%                  | 21-40%/10-20%          | 1-3%     | GATA2/SRSF2                  | NA                      | NA              |
| Dysplasia                    | 609948 | 83   | F   | 3.2  | 8.1  | 136 | <8%                | <10%                  | 10-20%/21-30%          | 1-3%     | ASXL1, RIMS1, U2AF1         | NA                      | NA              |
| Dysplasia                    | 658726 | 66   | F   | 6    | 9.6  | 887 | 10-20%<10%         | <10%                  | >10%                    | 1-3%     | None                        | NA                      | NA              |
| Dysplasia                    | 668295 | 79   | F   | 8.3  | 8.3  | 379 | <8%                | <10%/10-20%           | 21%-30%                 | 1-3%     | SF3B1, MUC16                 | NA                      | NA              |
| Dysplasia                    | 680791 | 59   | M   | 9.7  | 9.7  | 146 | 10-20%             | <10%                  | 21%-30%                 | 1-3%     | DST                        | NA                      | NA              |
| Dysplasia                    | 755644 | 65   | M   | 6    | 9.6  | 897 | <10%/10-20%        | <10%<10-20%           | 10-20%/21-30%          | 1-3%     | None                        | NA                      | NA              |
| Dysplasia                    | 796085 | 75   | M   | 6.6  | 11.5 | 45  | <8%                | <10%<10-20%           | 21%-30%<30-20%         | 1-3%     | LRPIB, TET2, SRSF2           | NA                      | NA              |
| Dysplasia                    | 831900 | 77   | M   | 1.7  | 9.6  | 21 | 21-50%/10-20%      | <10%<10-20%           | 10-20%/21-30%          | 1-3%     | ASXL1, CBF1, EZH2, S1, STAG2, TET2 | NA                      | NA              |
| Dysplasia                    | 858330 | 89   | M   | 2.5  | 8.6  | 79  | 10-20%<10%         | <10%/21-50%           | 10-20%                  | 1-3%     | CD8H, TRA2B                  | NA                      | NA              |
| Dysplasia                    | 884180 | 67   | F   | 2    | 10.8 | 72  | <8%                | 10-20%<30%            | <10%                    | 1-3%     | None                        | NA                      | NA              |
| Dysplasia                    | 932888 | 69   | M   | 13.4 | 11.9 | 60  | >50%/10-20%        | <10%<10-20%           | 10-20%/21-30%          | 1-3%     | SMCTA                       | NA                      | NA              |
| Consensus Morphologic Diagnosis | UPN   | Age | Sex | WBC  | Hgb | Plts | Myeloid Dysplasia* | Erythroid Dysplasia* | Megakaryocytic Dysplasia* | Blasts* | Genes with Somatic Mutations | Follow-Up interval (days) | Treated for MDS |
|-------------------------------|-------|-----|-----|------|-----|------|-------------------|----------------------|--------------------------|--------|-----------------------------|------------------------|---------------|
| Dysplasia                     | 977120| 54  | M   | 1.6  | 6.9 | 129  | <10%             | 10-20%               | 10-20%/21-50%           | 1-3%   | IDH1, SRSF2                  | NA                     | NA            |
| Dysplasia                     | 983847| 55  | F   | 6.5  | 7.9 | 404  | <10%             | 10-20%               | 21%-50%                 | 1-3%   | NA                          | NA                     | NA            |
| Equivocal Dysplasia           | 968547| 71  | F   | 2.5  | 11.9| 31   | <10%             | <10%/10-20%           | <10%                    | 1-3%   | BCR3, DNMT3A, EBB1, PHB, PRK2SF1, RUNX1, TET2 | 1252                   | Yes           |
| Equivocal Dysplasia           | 610864| 55  | F   | 2.9  | 9.3 | 34   | <10%             | <10%                  | <10%/10-20%             | 1-3%   | None                        | NA                     | No            |
| Equivocal Dysplasia           | 721979| 82  | M   | 3.9  | 9.2 | 174  | <10%/21-50%      | <10%                  | <10%/21-50%             | 1-3%   | PHS, TET2                   | 721                    | Yes           |
| Equivocal Dysplasia           | 724989| 84  | M   | 1    | 12.7| 22   | <10%             | 10-20%/-<10%          | <10%                    | 1-3%   | PKHD1, MAG2, CSMD3, DNMT3A, RAD21 | 21                    | Yes           |
| Equivocal Dysplasia           | 728125| 54  | M   | 1.7  | 7.4 | 46   | <10%             | 10-20%               | 21%-50%                 | 1-3%   | SF3B1, U2AF1                | 96                     | Yes           |
| Equivocal Dysplasia           | 786953| 81  | F   | 13.6 | 9.9 | 242  | <10%             | 10-20%/-<10%          | <10%-/>50%              | 1-3%   | CBL, TET2, U2AF1            | 646                    | Yes           |
| No Dysplasia                  | 863943| 63  | F   | 1.7  | 11.7| 36   | <10%             | <10%                  | <10%                    | 1-3%   | CMYC5                       | 1712                   | Yes           |
| No Dysplasia                  | 948042| 77  | F   | 7    | 9.6 | 449  | <10%             | <10%                  | <10%                    | 1-3%   | CEBPA                       | 980                    | No            |
| No Dysplasia                  | 532207| 65  | F   | 3.0  | 25  | 38   | <10%             | <10%                  | <10%                    | 1-3%   | TET2                        | 995                    | No            |
| No Dysplasia                  | 529198| 61  | F   | 3.4  | 8.2 | 327  | <10%             | <10%                  | <10%                    | 1-3%   | TET2                        | 995                    | No            |
| No Dysplasia                  | 577914| 48  | F   | 1.8  | 6.1 | 237  | <10%             | <10%                  | <10%                    | 1-3%   | TET2                        | 995                    | No            |
| No Dysplasia                  | 969282| 19  | M   | 1.4  | 8.5 | 24   | <10%             | <10%                  | <10%                    | 1-3%   | None                        | 596                    | No            |
| No Dysplasia                  | 859848| 54  | F   | 2.6  | 10.9| 40   | <10%             | <10%                  | <10%                    | 1-3%   | SF3B1                       | 473                    | No            |
| No Dysplasia                  | 976020| 63  | F   | 0.9  | 10.3| 288  | <10%             | <10%                  | <10%                    | 1-3%   | None                        | 480                    | No            |

White blood cell counts (WBC) reported in 10^3 cells/mcl
Hemoglobin (Hgb) reported in g/dl
Platelets (Plts) reported in 10^3/mcl
NA, Not Applicable

*For discordant cases, dysplasia data is listed as reviewer 1 findings/reviewer 2 findings.