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Risk of Disease Progression in Low-Risk MDS is Linked to Distinct Epigenetic Subtypes

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To the editor:

Myelodysplastic syndromes (MDS) present with aberrant promoter DNA hypermethylation, the extent of which correlates with disease aggressiveness (1, 2). Yet, it is unclear whether

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Authors' contributions:

TQ conceived and designed the study, performed data analysis and wrote the manuscript. JS performed experiments. RR, RLL and FTR performed experiments and analyzed data. VK designed the study and contributed key reagents. SDN conceived the study, contributed to study design and data interpretation. MEF conceived and designed the study, oversaw data analysis and interpretation and wrote the manuscript.

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extensive epigenetic abnormalities seen in more aggressive forms of MDS are present from the early stages of the disease, or whether they simply represent a consequence of disease progression. Low-risk MDS patients often require only supportive care with growth factor stimulation or red blood cell transfusions (3); however, some present with a rapidly progressive form of low-risk MDS, resulting in worsening cytopenias, increased transfusion requirement, or a spike in their bone marrow (BM) blasts without AML transformation, while others remain much longer in a relatively stable cytopenic phase. Currently, the Low Risk Prognostic Scoring System (LR-PSS) is the only prognostic scoring system specifically designed for low-risk MDS (4). Notably, when Bejar and colleagues later validated this scoring system, they determined that mutational status of EZH2, an epigenetic modifier, was an independent risk factor and that combining this information with the LR-PSS identified patients with poor prognosis (5). We sought to determine whether epigenetic profiles present on peripheral blood mononuclear cells (PB MNC) at diagnosis are correlated with clinical outcome, and whether there is accompanying epigenetic progression at the time of clinical progression. The study's ultimate goal was to determine whether this type of easily accessible samples, which do not require invasive studies or complex cell isolation strategies, could be harnessed for future development of clinical biomarkers to improve risk-stratification of low-risk MDS.

We studied a cohort of 20 patients, including 13 with stable MDS (no clinical or laboratory progression within 18 months of diagnosis) and 7 with progressive MDS (presenting with worsening cytopenias or increased transfusion requirement within 18 months of diagnosis), for which we had paired diagnostic and follow-up samples. There were no significant differences between the two groups with the exception of hemoglobin levels, which at diagnosis were on average ~1 g/dl higher in MDS patients with stable disease (11.7 vs. 10.4 g/dl, Wilcoxon rank-sum test, $p < 0.01$), a difference which accentuated at the time of progression (11.3 vs. 9.6 g/dl, Wilcoxon rank-sum test, $p < 0.01$) (Supplementary Table 1 and Supplementary Figure 1).

To fully characterize our cohort, we performed targeted sequencing of a panel of 59 genes frequently mutated at >5% frequency in myeloid malignancies. *TET2* and *RUNX1* were the most frequently mutated genes in our cohort. While the study was not powered to identify associations between individual mutations and clinical outcome, we did observe that the average number of mutations per patient at diagnosis was significantly higher in the progressive cases than in the stable MDS patients (Stable=1.2, Progressive=3.2, Wilcoxon Rank-sum test p-value=0.01) (Figure 1A and Supplementary Table 2), indicating that mutation burden may serve as an indicator for patients at higher risk for developing progressive bone marrow failure.

To determine whether specific DNA methylation profiles correlated with clinical outcome in low-risk MDS patients, we examined methylcytosine profiles using enhanced reduced representation bisulfite sequencing (ERRBS) (6). A direct comparison, based on the likelihood ratio test conditional on estimated group variance (7), showed distinct DNA methylation profiles at diagnosis that correlated with the clinical evolution, identifying 356 differentially methylated regions (DMRs) between progressive and stable low-risk MDS (absolute average methylation difference ≥25% and false discovery rate [FDR] <10%)

(Figure 1B and Supplementary Table 3). Approximately 2/3 of these DMRs were hypomethylated in patients who evolved with progressive disease relative to those who presented with stable disease. Notably, the methylation pattern observed in stable patients more closely resembled that seen in normal, healthy individuals at those same regions, whereas progressive patients presented with profiles highly divergent from normal. (Figure 1C and Supplementary Figure 2A).

Analysis of these DMRs to annotated genomic regions revealed that they were significantly enriched at intronic regions (DMRs 40% vs. BG 32%, binomial test $p = 0.001$) and at intergenic regions (DMRs 40% vs. BG 35%, $p = 0.02$), with depletion at promoter regions (DMRs 10% vs. Background [BG] 24%, $p = 1.66\times10^{-11}$) (Figure 1D). Moreover, DMRs were depleted from CpG islands (DMRs 12% vs. BG 29%, $p = 2.05\times10^{-15}$) but showed enrichment at CpG shores (DMRs 21% vs. BG 15%, $p = 0.002$). This pattern was conserved across both regions that were hyper- and hypomethylated at baseline in progressive cases relative to stable ones. Finally, using the ENCODE annotation of enhancers in hematopoietic cells (8) we performed enrichment analysis of DMRs relative to intra- and intergenic enhancers. Baseline DMRs between stable and progressive MDS were significantly enriched at both intra- (DMRs 23% vs. BG 19%, $p = 0.03$) and intergenic enhancers (DMRs 18% vs. BG 15%, $p = 0.05$); this was even more pronounced amongst 2/3 of the DMRs with lower methylation levels in progressive MDS relative to stable MDS (Figure 1D).

Analysis of specimens from the same patients either at the time of progression or after a minimum of 18 months follow-up for the stable cases revealed further epigenetic divergence, with DNA methylation profiles that more strongly segregate the progressive MDS patients from the stable MDS patients and normal healthy donors (Supplementary Figure 2B). Supervised analysis showed the number of DMRs was almost doubled at follow-up, with 681 DMRs, the majority of which were also hypomethylated in progressive MDS. (Figure 2A and Supplementary Table 4). While progressive samples became even more aberrantly methylated at the time of progression compared to normal controls, stable MDS continued to retain DNA methylation profiles closely resembling their healthy counterparts, similar to that observed at diagnosis (Figure 2B). Again, DMRs detected at the time of progression consisted predominantly of regions with less methylation in progressive than stable cases. These DMRs were significantly depleted at promoter regions (DMRs 13% vs. BG 23%, binomial test $p = 8.17\times10^{-11}$), an observation particularly true for the hypomethylated DMRs, which were instead significantly enriched at CpG shores (hypo-DMRs 31% vs. BG 15%, $p < 2.2\times10^{-16}$) and enhancers (hypo-DMRs 54% vs. BG 34%, $p < 2.2\times10^{-16}$). By contrast, hypermethylated DMRs were enriched at CpG islands (hyper-DMRs 36% vs. BG 28%, $p = 0.02$) (Figure 2C), indicating that despite marked progression, the nature of the epigenetic abnormality and the preferential targeting by aberrant methylation of regulatory regions remains similar at both time points.

Finally, we performed gene set enrichment analysis (GSEA) (9) using the KEGG database to identify biological pathways differentially regulated in stable vs. progressive MDS. This analysis revealed that differentially methylated genes between stable and progressive MDS at diagnosis were enriched in pathways that included oxidative phosphorylation (Nominal Enrichment Score [NES]: 2.39, FDR q-value: 0.005), cytokine-receptor interaction (NES:

2.34, FDR q-value: 0.006), p53 signaling (NES: 2.32, FDR q-value: 0.004), WNT signaling (NES: 2.03, FDR q-value: 0.025), mTOR signaling (NES: 2.01, FDR q-value: 0.03), nucleotide excision repair (NES: 1.99 and FDR q-value: 0.03) and DNA replication (NES: 1.95 and FDR q-value: 0.03) (Figure 2D and Supplementary Table 5). Notably, upon progression, KEGG pathway enrichment analysis showed a more significant enrichment of WNT signaling (NES: 3.22, FDR q-value: <0.00001), indicating that this pathway is more strongly compromised at disease progression (Supplementary Table 6). Moreover, additional pathways relevant to MDS biology and risk of progression were also enriched at the time of progression including spliceosome (NES: 3.68, FDR q-value: <0.00001), cell cycle (NES: 3.26, FDR q-value: <0.00001), MAPK signaling (NES: 2.4, FDR q-value: 0.003) and several cancer-related pathways such as pathways in cancer (NES: 3.69, FDR q-value: <0.00001) and chronic myeloid leukemia (NES: 2.37, FDR q-value: 0.003) (Figure 2D).

In summary, we report the existence of distinct DNA methylation profiles at diagnosis despite the fact that these patients show almost no additional distinguishable clinical or laboratory differences. These findings have potential clinical implications since PB MNC epigenetic differences at diagnosis may have the potential to be harnessed to develop predictive biomarkers that can be performed on an easily obtained specimen, without requiring additional bone marrow aspirates, time-consuming cell sorting or magnetic fractionation steps. We expect that extending our study to a larger cohort will result in the identification of an extended, robust set of DMRs between stable and progressive MDS and that confirmed DMRs can serve as candidate biomarkers to distinguish progressive MDS from stable cases at diagnosis. Such a study could be easily implemented with PB samples obtained during routine follow-up of low-risk MDS patients. Biomarkers identified in a larger study would allow for closer monitoring and earlier intervention in cases predicted to behave with rapid progression to bone marrow failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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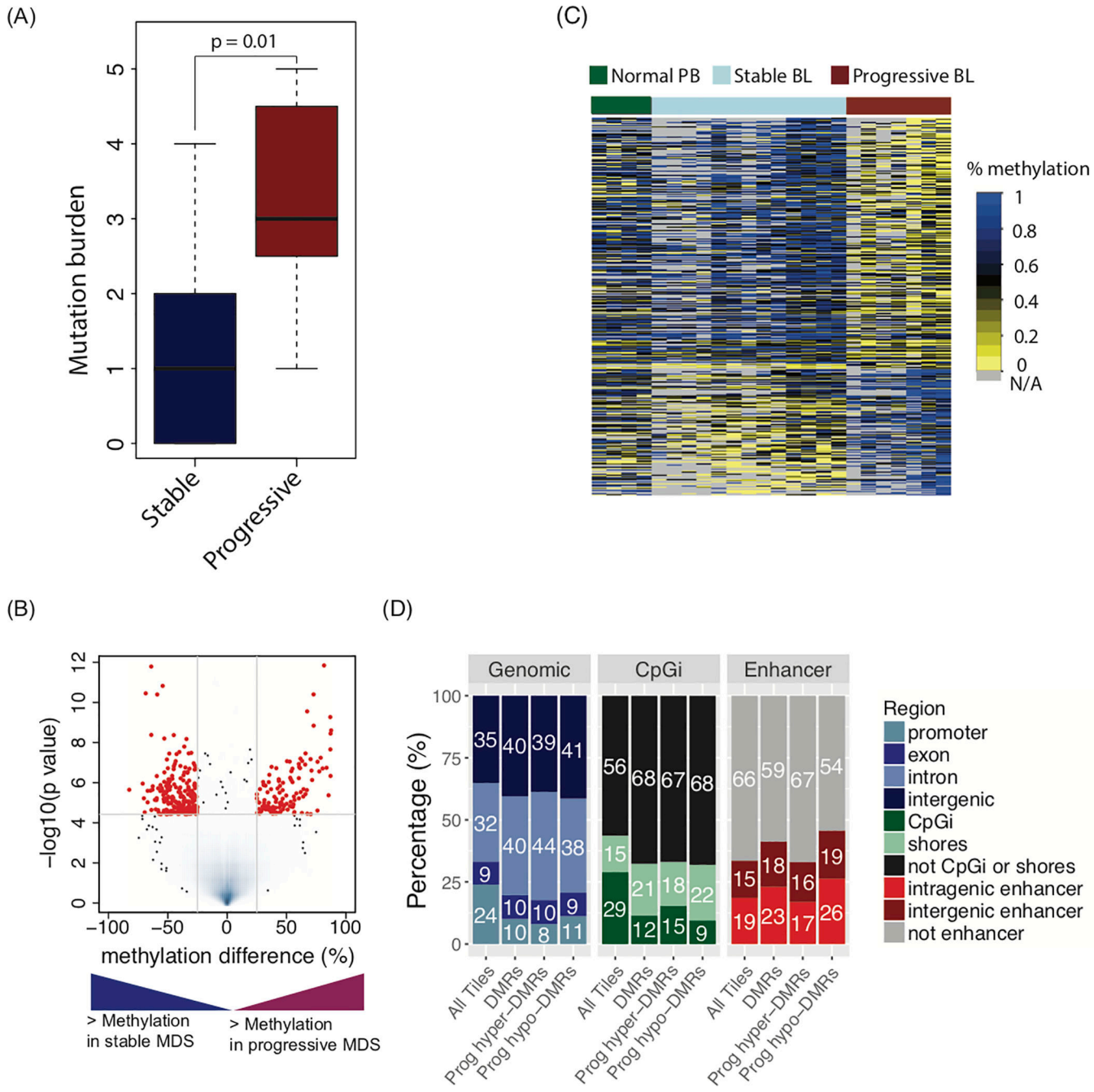

Figure 1. Distinct methylation profiles characterize patients with stable and progressive low-risk MDS at diagnosis.

(A) Boxplot comparing mutation burden at diagnosis in stable and progressive MDS patients. **(B)** Volcano plot illustrating methylation difference between 7 progressive and 13 stable MDS patients at diagnosis. Mean methylation difference between the two groups is represented on the x-axis and statistical significance (-log10p value) on the y-axis. Red dots indicate 386 significant differentially methylated regions (DMR) **(C)** Heatmap illustrating the percentage methylation of the baseline DMRs (n=386) across the different comparative groups. Each row represents a DMR, and each column represents an individual sample

(Normal PB: normal peripheral blood, Stable BL: stable low-risk MDS at baseline and Progressive BL: progressive low-risk MDS at baseline). **(D)** Stacked bar charts illustrate the relative proportion of all CpG tiles captured by the ERRBS assay and the identified DMRs annotated to RefSeq gene promoter, exonic, intronic, and intergenic regions (left); to CpG islands, CpG shores and regions beyond CpG shores (middle); and to inter- and intra-genic enhancers (right). Prog hyper-DMR: hyper-methylated DMRs in progressive patients compared with stable patients; Prog hypo-DMR: hypo-methylated 638 DMRs in progressive patients compared with stable patients.

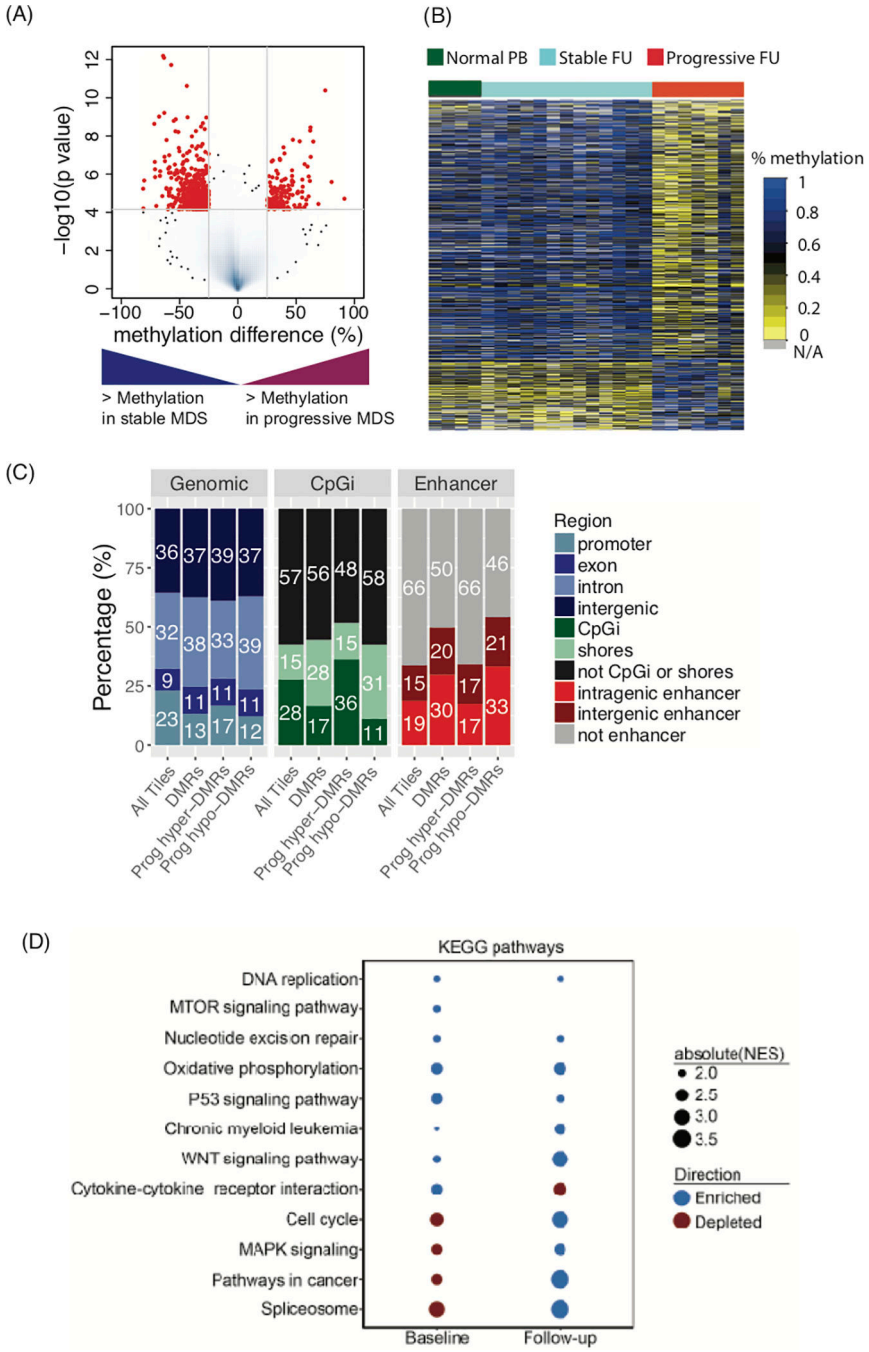

Figure 2. Clinical progression in low-risk MDS is accompanied by epigenetic progression.

(A) Volcano plot illustrating methylation difference between 7 progressive and 13 stable MDS patients at follow-up. Mean methylation difference between the two groups is represented on the x-axis and statistical significance (-log10p value) on the y-axis. Red dots indicate 681 significant differentially methylated regions (DMR) **(B)** Heatmap illustrating the percentage methylation of the follow-up DMRs (n=681) across the different comparative groups. Each row represents a DMR, and each column represents an individual sample (Normal PB: normal peripheral blood, Stable FU: stable low-risk MDS at follow-up and

Progressive FU: progressive low-risk MDS at follow-up). **(C)** Stacked bar charts illustrate the relative proportion of all CpG tiles captured by the ERRBS assay and the identified DMRs annotated to RefSeq gene promoter, exonic, intronic, and intergenic regions (*left*); to CpG islands, CpG shores and regions beyond CpG shores (*middle*); and to inter- and intra-genic enhancers (*right*). Prog hyper-DMR: hyper-methylated DMRs in progressive patients compared with stable patients; Prog hypo-DMR: hypo-methylated DMRs in progressive patients compared with stable patients. **(D)** Bubble plots illustrating enrichment of DMRs between stable and progressive low-risk MDS at select KEGG pathways at baseline (left) and follow-up (right).