Transcriptomic Signatures of Hypomethylating Agent Failure in Myelodysplastic Syndromes and Chronic Myelomonocytic Leukemia
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Hypomethylating agents (HMAs) are the standard of care for myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CMML). HMA treatment failure is a major clinical problem and its mechanisms are poorly characterized. We performed RNA sequencing in CD34+ bone marrow stem hematopoietic stem and progenitor cells (BM-HSPCs) from 51 patients with CMML and MDS before HMA treatment and compared transcriptomic signatures between responders and nonresponders. We observed very few genes with significant differential expression in HMA non-responders versus responders, and the commonly altered genes in non-responders to both azacitidine (AZA) and decitabine (DAC) treatments were immunoglobulin genes. Gene set analysis identified 78 biological pathways commonly altered in non-responders to both treatments. Among these, we determined that the γ-aminobutyric acid (GABA) receptor signaling significantly affected hematopoiesis in both human BM-HSPCs and mice, indicating that the transcriptomic signatures identified here could serve as candidate biomarkers and therapeutic targets for HMA failure in MDS and CMML. Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

HIGHLIGHTS

- A transcriptomic HMA resistance signature was obtained in a patient with CD34+ BM HSPCs.
- Commonly downregulated genes in AZA and DAC non-responders were immunoglobulins.
- Biological pathways in AZA and DAC non-responders were mostly drug specific.
- Non-responders to AZA and DAC therapies shared 78 altered pathways in BM HSPCs.
- GABA signal, upregulated in AZA and DAC non-responders, affected BM hematopoiesis.

Myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CMML) are heterogeneous myeloid neoplastic disorders characterized by ineffective hematopoiesis leading to cytopenias and an increased risk of progression and transformation to acute myeloid leukemia (AML) [1]. The hypomethylating agents (HMAs) azacitidine (AZA) and decitabine (DAC) have been reported to improve the natural history of patients with MDS and CMML [2] and remain standard therapies in the treatment of MDS [3]. However, a majority of patients experience primary HMA failure (no response or progression during treatment) or secondary failure (relapse after initial response) [4,5]. Following treatment failure, the median survival for these patients is only 4–6 months [4,5].

A molecular biomarker that could predict primary HMA failure before the start of treatment would allow for the selection of alternative treatments. Biological abnormalities such as mutations in the Ten-eleven translocation 2 (TET2) gene [6], patterns of differentially methylated DNA regions (DMR) [7], and aberrations at the cellular level, such as the enzymes or transporters involved in azanucleoside metabolism [8], have been reported to contribute to HMA resistance in myeloid neoplasms. To date, however, the precise mechanisms underlying HMA resistance and treatment failure remain poorly understood.

Compared with mutation and methylation analysis, the strategy of using transcriptomic sequencing to study altered gene expression and biological pathways in patients with MDS and CMML, especially in
their bone marrow hematopoietic stem and progenitor cells (BM HSPCs), which are associated in HMA failure, has not been widely reported [9]. In this study, we conducted RNA sequencing (RNA-Seq)-based transcriptomic analysis in baseline CD34+ BM HSPCs collected from MDS and CMML patients before HMA therapy. Gene expression and associated biological signaling pathways were compared between primary non-responders and responders to HMA-based treatments. Subsequently, the hematopoietic effect and therapeutic potential of a representative transcriptomic signature signaling pathways identified in HMA non-responders were determined in both in vitro and in vivo biological experiments.

**METHODS**

**Patient Sample Collection**

Bone marrow aspirates were collected from patients before HMA treatment. By use of morphologic, cytochemical, and immunohistochemical analysis along with classification according to World Health Organization (WHO) criteria, diagnosis was confirmed by an expert hematopathologist (RKS) at the University of Texas MD Anderson Cancer Center (MDACC). Informed consent was collected according to institutional guidelines in accordance with the Declaration of Helsinki. BM mononuclear cells (MNCs) were enriched using Ficoll (GE Healthcare, Chicago, IL), according to the manufacturer’s instructions. BM HSPCs were enriched using magnetic cell separation and CD34+ magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany).

Responders were defined as patients with hematological improvement (HI), complete remission (CR), marrow CR, or partial remission (PR) after four to six cycles of treatment with either DAC or AZA. Nonresponders were defined as having stable disease, progression to AML (≥20% BM blasts), or no evidence of response using the International Working Group (IWG) 2006 criteria [10].

**RNA Sequencing Analysis**

RNA from sorted BM CD34+ cells was isolated using the TRIzol RNA Isolation Kit (Fisher Scientific, Waltham, MA) followed by RNA-Seq library construction. FASTQ files were processed in TopHat2 using the default options [11]. Differential gene expression analysis (DESeq) was conducted using DESeq2 with cutoff at a false discovery rate (FDR) <0.1 for statistical significance [12]. Pathway enrichment analysis was performed using gene set enrichment analysis (GSEA) [13], with the fsgsea library in R [13]. The t-statistics from the differential expression analysis were used to rank the genes for this analysis. Twenty thousand gene permutations were used to calculate statistical significance, and an FDR <0.25 was considered for statistical significance of a gene set.

**Methylcellulose Culture of Primary BM CD34+ Cells and Colony Formation Unit Assay**

CD34+ cells isolated from healthy individuals (AllCells, Alameda, CA) and from patients with MDS or CMML were plated into human methylcellulose colony formation medium Methocult-4434 (Stem Cell Technology, Vancouver, BC, CA) to culture for 10–14 days before the counting of colonies and cell collection. Collected cells were analyzed by flow cytometry using a Gallios flow cytometer (Beckman Coulter, Indianapolis, IN). For treatment, baclofen (5 mmol/L), CGP52432 (5 mmol/L), or AZA (200 nmol/L) was added into Methocult-4434 medium. For flow cytometry analysis, the antibodies used were human CD71-fluorescein isothiocyanate (FITC), human CD235a−phycocerythrin (PE), AnnexinV−allophycocyanin (APC), and Live/Dead Fixable Aqua (Thermo Fisher Scientific, Wal-tham, MA).

**Treatment and Analysis of Mice**

C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were maintained in an animal facility. Animal care followed MDACC Institutional Animal Care and Use Committee oversight and guidelines. Mice around 10 weeks old were treated with baclofen (Millipore Sigma, St. Louis, MO) at the dose of 3 mg/kg body weight or with phosphate-buffered saline vehicle control (0.2 mL, i.p.) daily for 1 week. Mice were then euthanized with CO2, and BM cells were isolated. BM cells were analyzed by flow cytometry using a Gallios flow cytometer (Beckman Coulter, Indianapolis, IN). The Lin−/Sca1+/-/ cKit+ (LSK) BM cells were isolated by fluorescence-activated cell sorting (FACS). Isolated LSK cells were plated into mouse Methocult-3344 (STEM Cell Technology) to culture for 10–14 days before the counting of colonies. The antibodies used for mouse BM cell analysis and LSK isolation included biotin mouse lineage cocktail (Miltenyi 130-090-858), streptavidin−APC−C7 (554063, BD Pharmingen, East Rutherford, NJ), cKit−APC (553356, BD Pharmingen), Sca1−PerCP (45-5981-82, eBioscience, San Diego, CA), CD71−FITC (11-0314-85, eBioscience), and Ter119-eFluor450 (48-5921-82, eBio-science).

**RESULTS**

**Clinical and Genetic Characteristics of Patients**

We collected the CD34+ BM HSPCs from 51 patients before their HMA treatments. After sample collection, 35 patients were treated with AZA and 16 were treated with DAC. The clinical characteristics of the patients are listed in Table 1. Their HMA treatments and primary responses are listed in Table 1 and also illustrated in Figure 1.

Among the 17 patients with CMML, the median age was 74 years (range: 59–87 years), and 24% of them were female. Approximately 24% of the patients had a prior malignancy, of whom 25% had prior chemotherapy exposure and 50% had prior radiotherapy (XRT). According to the Revised International Prognostic Scoring System (IPSS-R) [11], patients were classified into the very low (18%), low (29%), intermediate (18%), high (24%), and very high (12%) categories. Median (range) values of BM blasts, white blood cell (WBC) counts, hemoglobin levels, platelets, and absolute neutrophil counts (ANCs) were 5% (1%–17%), 9.4 (2.1–67) × 10^9/L, 10.8 (8.4–14.6) g/dL, 67.5 (16–160) × 10^9/L and 5 (0.96–34.2) × 10^9/L, respectively. Fifty-nine percent of all patients were treated with single-agent AZA or AZA in combination (Table 1), and the remainder were treated with single-agent DAC including 5 patients with DAC and 1 with ASTX727 (oral decitabine) or DAC in combination (Table 1).

All 34 patients with MDS were diagnosed with MDS with excess blasts (MDS-EB). The median age was 70 years (range: 47–87 years) and 26% were female. Twenty-six percent had a prior malignancy, 33% of whom have had prior chemotherapy and 22% prior XRT. Patients were classified into intermediate (24%), high (32%), and very high subgroups (44%) with the IPSS-R [11].
The median (range) percentage of BM blasts, WBC counts, hemoglobin values, platelet counts, and ANCs were 10.5% (5%–18%), 2.3 (0.6–9.8) £ 10^9/L, 9.3 (6.9–15) g/dL, 76.5 (11–325) £ 10^9/L, and 0.8 (0.2–3.5) £ 10^9/L respectively. Seventy-four percent of patients were treated with either AZA as a single agent or AZA in combination, and the remainder were treated with DAC (including 4 patients with ASTX727, 3 with DAC, and 2 with guadecitabine).

On the basis of Student t tests for continuous variables such as age, BM blast percentage, WBC, hemoglobin, platelets, and ANCs; Fisher exact tests for categorical variables such as sex, prior malignancy, and treatment; and χ^2 tests for comparing IPSS-R and cytogenetic levels defined by IPSS-R, none of the variables were found to be significantly associated with patients’ responses to HMA treatments (Table 1). We also analyzed the effects of the most frequent somatic mutations in MDS and CMML (based on targeted next-generation
sequencing in BM mononuclear cells) on HMA responses of 41 patients in the cohort (Supplementary Table E1 and Supplementary Figure E1A, online only, available at www.exphem.org). Results indicated no significant effects from these common mutations on treatment responses (Supplementary Figure E1B).

Analysis of Differentially Expressed Genes in AZA and DAC Nonresponders

Next, transcriptomic signatures obtained in the BM CD34+ cells of patients from the AZA and DAC cohorts were analyzed separately, and the MDS and CMML patients in each cohort were combined because of the relatively small sample size.

In the AZA cohort (14 non-responders and 21 responders; Figure 1 and Table 1), DESeq analysis indicated that only 25 genes (0.075% of the 30 x 10^3 genes reported by RNA-Seq) had significantly different levels of RNA expression (FDR <0.1 and fold change >1.5) when responders and nonresponders were compared. Among these 25 genes, 15 (60%) were expressed at higher levels whereas 10 (40%) were expressed at lower levels in AZA nonresponders (Supplementary Figure E2 and Supplementary Table E2, online only, available at www.exphem.org). Of note, all 10 genes with lower expression in nonresponders encoded immunoglobulin proteins (Figure 2).

In the DAC cohort (6 nonresponders and 10 responders, Figure 1 and Table 1), DESeq results indicated that 152 genes (0.45% of the total number of genes reported by RNA-Seq) had significantly different levels of RNA expression (FDR <0.1 and fold change >1.5) when responders and nonresponders were compared. Among these 152 genes, 114 genes (75%) were expressed at higher levels and 38 genes (25%) at lower levels in nonresponders (Supplementary Figure E3A and B, online only, available at www.exphem.org; Supplementary Table E2). Similar to the observations made in the AZA cohort, 7 of the 38 genes (18%) with lower expression in DAC nonresponders encode immunoglobulin proteins (Figure 2; Supplementary Table E3, online only, available at www.exphem.org).

When genes with significantly altered expression in AZA and DAC nonresponders were compared, there was no genes commonly upregulated in nonresponders to both of these treatments. Two genes were commonly downregulated in both AZA and DAC nonresponders: IGV3-20 and IGLV3-21 (Figure 2). Of interest, both of these genes encode immunoglobulins.

Overall, these analyses indicate that differential expression of individual genes associated with AZA and DAC treatment failure in baseline MDS-EB and CMML patients are very rare (<1% of all genes detected by RNA-Seq), and only a few immunoglobulin genes were commonly downregulated in both AZA and DAC non-responders.

Analysis of Differentially Regulated Biological Signaling Pathways in AZA and DAC Nonresponders

We next studied biological signaling pathways differentially altered in HMA nonresponders via Gene Set Enrichment Analysis (GSEA) of the RNA-Seq data. Based on the REACTOME pathway database [14], we identified 257 pathways that were significantly differentiated (FDR <0.25) between AZA nonresponders and responders (Supplementary Table E4, online only, available at www.exphem.org), and 381 pathways that significantly differentiated between DAC nonresponders and responders (Supplementary Table E5, online only, available at www.exphem.org). When comparing the altered pathways between AZA and DAC nonresponders, a majority of pathways were AZA or DAC specific. For instance, the interferon and toll-like receptor pathways were significantly upregulated in DAC
nonresponders but not in AZA nonresponders. Only 78 pathways (20%–30% of all significantly regulated REACTOME pathways) were commonly altered in both AZA and DAC nonresponders (Figure 3; Supplementary Table E6, online only, available at www.exphem.org).

Among these 78 pathways commonly altered in both AZA and DAC nonresponders, upregulated pathways were clustered mainly in four biological functions: neurotransmitters including olfactory and associated G-protein receptor signals; O-linked glycosylation signals; cell junction organization signals; and solute carrier (SLC)-mediated transmembrane transport signals (Figure 3, Supplementary Table E6). Downregulated signals were mainly associated with four biological functions: translation regulatory signals involving ribosomal proteins; immune regulatory signals involving immunoglobulins; mitochondria respiratory chain signals; and trans-Golgi transportation (Figure 3; Supplementary Table E6). These results indicated that there are

Figure 2 Downregulated genes in DAC and AZA nonresponders. Significantly downregulated genes (FDR <0.1, fold change >1.5) in nonresponder versus responder MDS-EB and CMML patients before DAC and AZA treatment with immunoglobulin and variant genes are colored in red. AZA=azacytidine; CMML=chronic myelomonocytic leukemia; CR=complete response; DAC=decitabine; FDR=false discovery rate; MDS-EB=myelodysplastic syndrome with excess blasts.

Figure 3 Normalized enrichment scores for commonly altered REACTOME [14] pathways in both the AZA and DAC treatment groups. Pathways upregulated in nonresponders were classified into clusters of Neurotransmitter, O-Linked Glycosylation, SLC-Mediated Transmembrane, Cell Junction Organization, and Others, while pathways downregulated in nonresponders were classified into the clusters Translation Regulatory Signals, Immune Regulatory Signals, Respiratory Chain Signals, Trans-Golgi Transportation, and Others. AZA=azacytidine; DAC=decitabine; SLC=solute carrier.
Characterization of γ-Aminobutyric Acid Neurotransmitter Receptor Signaling in MDS and CMML

We next determined to characterize the signature signaling pathways shared between AZA and DAC nonresponders for their biological effects on hematopoiesis and translational potentials. Activation of GABA signaling is one of the pathways significantly upregulated in both AZA and DAC nonresponders based on transcriptomic analysis (Figure 3; Supplementary Table E6). Importantly, several recent studies reported the expression of GABA receptors in BM HSPCs [15,16], implicating involvement of this neurotransmitter signal in the regulation of hematopoiesis.

We surveyed an RNA-Seq data set obtained from BM CD34+ cells of treatment-naive MDS (N = 24), CMML (N = 19), and healthy donors (N = 9) [17]. Among the 21 human GABA receptor subunit genes, the GABAB receptor subunit-1 gene GABBR1 was the only one to be significantly overexpressed in BM CD34+ cells of patients with MDS and CMML compared with healthy donor controls (Supplementary Figure E4A, online only, available at www.exphem.org). Of note, among all GABA receptor genes, GABBR1 also had a comparably higher RNA expression level in BM CD34+ cells of healthy individuals (Supplementary Figure E4A). Consistently, flow cytometry analysis indicated that GABAB receptor protein was expressed on the cell surface of both whole BM and CD34+ cell populations, with increased levels in MDS or CMML than in healthy donors (Supplementary Figure E4B,C). To study if activation of GABAB receptor activation affects hematopoietic function of BM HSPCs, we applied the GABAB receptor agonist baclofen to cultured primary BM CD34+ cells isolated from heathy donors (N = 6) and performed colony formation unit (CFU) analysis. Results indicated that baclofen significantly reduced the number of colonies formed by BM CD34+ cells (Figure 4A). When the cells collected from colonies in CFU assays were analyzed by flow cytometry analysis, results indicated that the frequency of CD71+/CD235a- erythroid cells was significantly decreased in baclofen-treated plates (Figure 4B). We next evaluated if GABAB receptor agonist could also affect hematopoiesis in vivo. Wild-type C57BL/6j mice were treated with baclofen (30 mg/kg body weight) or vehicle control for 1 week followed by BM cell analysis. Consistent with the in vitro observations made in CFU assays of human BM CD34+ cells, mice treated with baclofen exhibited a significantly reduced frequency of the CD71−/CD235a+ erythroid colonies (Figure 4D; Supplementary Figure E5B, online only, available at www.exphem.org) compared with the vehicle control and single-agent AZA-treated groups. Of note, all 4 patients had anemia with decreased hemoglobin counts (Supplementary Table E7, online only, available at www.exphem.org). This result suggests that inhibition of GABAB receptor signaling has the potential to improve erythropoiesis when combined with AZA in MDS.

DISCUSSION

The highly heterogeneous pathophysiology of myeloid neoplasms and poorly understood mechanisms underlying therapeutic action of HMAs pose substantial challenges to the understanding of the biology of HMA failure in MDS and CMML [18].

Although several studies have evaluated the effect of specific somatic mutations in response to HMA therapies in MDS and CMML, results have so far been inconclusive [6,19]. To date, only a few of these studies have been able to link differential DNA methylation with clinical responses to HMA treatment [7,20]. Compared with these strategies, RNA-Seq-based genome-wide transcription analysis in primary BM HSPCs of HMA treatment non-responders and responders has not been sufficiently applied to the investigation of biomarkers of HMA failure, partially because of the limited availability of BM HSPC samples from patients. Currently available reports on transcriptomic markers of HMA failure in BM HSPCs of MDS and CMML were obtained mainly from sample cohorts that were small or heterogeneous in cell types [9,21]. In comparison, RNA-Seq-based transcriptomic analysis reported in current work was performed in a comparable larger cohort with 51 patients before HMA therapies and all in purified CD34+ BM HSPCs. Baseline transcriptomic profiles of HMA treatment non-responders versus responders were compared to obtain the transcriptomic signatures of AZA and DAC nonresponders separately. AZA- or DAC-specific biomarkers, as well as biomarkers commonly associated with treatment failure of both drugs, were identified. Subsequent in vitro and in vivo biological studies of GABA receptor signaling, a pathway identified to be commonly upregulated in AZA and DAC nonresponders, further demonstrated the biological and translational values of the transcriptomic signatures revealed in this work.

Transcriptomic analysis of the CD34+ BM HSPCs analyzed in this study revealed the following major findings. First, significant differential expression of individual genes in HMA nonresponders versus responders was very rare, which constituted 0.075% and 0.45% of the total number of genes characterized by RNA-Seq for AZA and DAC nonresponders, respectively. Of interest, in the limited number of differentially expressed genes found in HMA non-responders, all genes downregulated in AZA nonresponders and a major proportion of downregulated genes in DAC non-responders encoded for immunoglobulins. This finding was further supported by GSEA pathway analysis, which revealed that more than 40% of the biological signaling pathways...
commonly downregulated in both AZA and DAC nonresponders were from the immune regulatory pathways with immunoglobulins as leading edges. Similar observations were also made when the transcriptomic analysis in the same cohort was performed by merging treatments of AZA and DAC but separating MDS and CMML, which identified 224 and 54 genes with significant differential expression between HMA responders and nonresponders in MDS-EB and CMML, respectively. Only 5 genes, all of which encode immunoglobulins, were commonly altered in MDS and CMML cohorts, and all were immunoglobulin genes (Supplementary Figures E6 and E7 and Supplementary Tables E8 and E9, online only, available at www.exphem.org).

In contrast to the rarity of differentially expressed genes between HMA nonresponders and responders, based on leading edge genes, more than 200 and 300 biological signaling pathways were identified by GSEA analysis to be associated with AZA and DAC treatment failure, respectively. Of note, most of the pathways identified in nonresponders were AZA or DAC specific, implicating different underlying biological mechanisms of AZA and DAC failure. There were 78 pathways commonly altered in nonresponders of both therapies. These differential pathways were clustered into several functional groups that have been implied to have roles in BM HSPC regulation. For instance, neurotransmitter and olfactory pathway-associated G protein-coupled receptor signals, commonly identified to be upregulated in AZA and DAC nonresponders, were recently reported to play a role in regulating the maintenance and differentiation of BM HSPCs [15]. Cell junction signaling was also commonly upregulated in AZA and DAC nonresponders, and consistently, the signaling involving cell junction component integrin was recently reported by Unnikrishnan et al. [9] to be associated with AZA resistance in MDS and CMML. Among the commonly downregulated biological signaling clusters in AZA and DAC nonresponders, one cluster was associated with the regulation of protein translation,
which involves many ribosomal protein genes. Consistently, it has been determined that a decrease in ribosomal proteins, such as RPS14, and the related impairment of ribosomal function constitute an important mechanism in MDS and CMML development [22,23].

Subsequent to the transcriptomic analysis and identification of common transcriptomic signature pathways that were associated with HMA failure, we provided further experimental evidence revealing the hematopoietic effect of GABA signaling, which was a commonly upregulated signature pathway in AZA and DAC nonresponders and belonged to the neurotransmitter functional cluster. We identified that the GABAB receptor subunit-1 gene GABBR1 was significantly overexpressed in BM CD34+ cells from patients with MDS and CMML, which was consistent with a prior finding observed by Steidl et al. [16] by cDNA arrays. More importantly, by using a potent GABA receptor agonist baclofen in both primary BM CD34+ cells and mice, we observed that activation of the GABAB receptor compromised hematopoiesis, particularly negatively affecting erythropoiesis. Consistently, another GABA receptor A subunit gene, GABRA1, has previously been reported to be involved in the regulation of BM megakaryocyte differentiation [15]. Finally, in cultured BM CD34+ cells obtained from patients with MDS, including MDS with HMA failure, we provided evidence that combining the GABAB receptor inhibitor CGP52432 with AZA could improve the formation of erythroid colonies in CFU assays. These observations further support the functional and translational relevance of the pathways identified in transcriptomic analysis.

We acknowledge that our study has several limitations. First, although the patient cohort in this study was comparably larger than most previously reported cohorts also analyzing BM HSPC from patients to study HMA failure, this sample size (N = 51) still limits the statistical power needed to generate robust associations. In addition, the HMA therapies in this study included not only single-agent HMA, but also combinations of HMAs and other drugs with different doses and treatment times that might affect the results of the analyses. Second, because of the sample size, when transcriptomic analysis was performed by separating AZA and DAC, we merged MDS with CMML patients. To investigate the potential interference arising from clinical differences between these two distinct entities, we further replicated the analysis by separating MDS and CMML (Supplementary Figures E6 and E7 and Supplementary Tables E8 and E9). Results obtained by merging treatments for each disease stated similar inferences. Finally, detailed molecular mechanisms underlying the regulation of hematopoiesis by GABAB receptor signaling and its interaction with AZA should be investigated in future work.

Data Availability

The data generated in this study are available upon reasonable request from the corresponding author.

Conflict of Interest Disclosure

The authors declare no competing interests.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2022.09.002.

REFERENCES


