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SHORT REPORT

Haematological Malignancy – Biology

DNA methylation profiling of myelodysplastic syndromes and clinical response to azacitidine: A multicentre retrospective study

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Summary

Real-world data have revealed that a substantial portion of patients with myelodysplastic syndromes (MDS) does not respond to epigenetic therapy with hypomethylating agents (HMAs). The cellular and molecular reasons for this resistance to the demethylating agent and biomarkers that would be able to predict the treatment refractoriness are largely unknown. In this study, we shed light on this enigma by characterizing the epigenomic profiles of patients with MDS treated with azacitidine. Our approach provides a comprehensive view of the evolving DNA methylation architecture of the disease and holds great potential for advancing our understanding of MDS treatment responses to HMAs.

KEYWORDS
azacitidine, DNA methylation, hypomethylating agents, myelodysplastic syndromes

Aleix Noguera-Castells and Ignacio Campillo-Marcos contributed equally.

INTRODUCTION

Myelodysplastic syndromes (MDS) comprise a family of myeloid malignancies with heterogeneous genotypes and phenotypes, which are characterized by ineffective haematopoiesis and an increased risk of progression to acute myeloid leukaemia (AML).[1–3] Genome-wide analyses have identified mutations in several DNA methylation regulators, such as *TET2*, *DNMT3A*, *IDH1* or *IDH2*.[1–3] Additionally, DNA methylation studies have unveiled an aberrant methylation pattern in MDS,[4] revealing the important disruption of the epigenetic landscape that occurs in these syndromes. To tackle the aberrant epigenomic profile, hypomethylating agents (HMAs), such as azacitidine, are used in the clinics to treat higher risk patients with MDS, leading to an increased overall survival rate.[1–3] However, a major limitation exists, such only 40%–50% of the higher risk patients respond to the treatment.[1–3] Although several potential biomarkers of azacitidine response in MDS have been

TABLE 1 Characteristics of the studied MDS patients.

Characteristics	Entire cohort ($n = 43$)	Discovery cohort ($n = 31$)	Validation cohort ($n = 12$)	Discovery versus validation p-value*
Sex				
Female	15	10 (32.3%)	5 (41.7%)	0.723
Male	28	21 (67.7%)	7 (58.3%)	
Age (years)				
<70	14	10 (33.3%)	4 (33.3%)	1.000
≥70	28	20 (66.7%)	8 (66.7%)	
MDS WHO 2017 subtypes				
MDS-EB1	15	10 (32.3%)	5 (41.7%)	0.924
MDS-EB2	16	12 (38.7%)	4 (33.3%)	
MDS-MLD	8	6 (19.4%)	2 (16.7%)	
MDS-SLD	2	2 (6.5%)	0 (0.0%)	
MDS-U	2	1 (3.2%)	1 (8.3%)	
Bone marrow blast count at diagnosis (%)				
<5%	12	9 (29.0%)	3 (25.0%)	1.000
5%–9%	16	11 (35.5%)	5 (41.7%)	
10%–19%	15	11 (35.5%)	4 (33.3%)	
IPSS-R cytogenetic risk[a]				
Very good	0	0 (0.0%)	0 (0.0%)	0.333
Good	11	10 (33.3%)	1 (8.3%)	
Intermediate	11	6 (20.0%)	5 (41.7%)	
Poor	7	5 (16.7%)	2 (16.7%)	
Very poor	13	9 (30.0%)	4 (33.3%)	
IPSS-R risk group[b]				
Very low (0–1.5)	0	0 (0.0%)	0 (0.0%)	0.380
Low (2–3)	2	2 (6.7%)	0 (0.0%)	
Intermediate (3.5–4.5)	7	5 (16.7%)	2 (16.7%)	
High (5–6)	13	11 (36.7%)	2 (16.7%)	
Very high (>6)	20	12 (40.0%)	8 (66.7%)	
Response to azacitidine[c]				
Responder	25	18 (58.1%)	7 (58.3%)	1.000
Non-responder	18	13 (41.9%)	5 (41.7%)	

Abbreviations: IPSS-R, International Prognostic Scoring System-Revised; MDS, myelodysplastic syndromes; MDS-EB1, MDS with excess blasts type 1; MDS-EB2, MDS with excess blasts type 2; MDS-MLD, MDS with multilineage dysplasia; MDS-SLD, MDS with single lineage dysplasia; MDS-U, MDS Unclassifiable; WHO, World Health Organization.

[a]Schanz J, Tüchler H, Solé F, Mallo M, Luño E, Cervera J, et al. New comprehensive cytogenetic scoring system for primary myelodysplastic syndromes (MDS) and oligoblastic acute myeloid leukaemia after MDS derived from an international database merge. J Clin Oncol 2012;30(8): 820-9.

[b]Greenberg PL, Tuechler H, Schanz J, Sanz G, Garcia-Manero G, Solé F, et al. Revised international prognostic scoring system for myelodysplastic syndromes. Blood 2012;120(12): 2454-65.

[c]Response criteria and the pertinent references are described in Data S1. Available data are shown.

*p-values were calculated using Fisher's exact test or chi-squared test for dichotomous or categorical respectively. p-values under 0.05 represent statistically significant association between co-variables.

proposed,[1–3] a comprehensive global profile of the DNA methylome in HMA-treated MDS longitudinal DNA samples before and after treatment has not been reported. Herein, we describe the consecutive DNA methylation changes that take place in patients with MDS upon HMA therapy according to clinical response, and identify a DNA methylation signature to potentially predict the efficacy of the treatment at diagnosis.

PATIENT SELECTION/STUDY DESIGN

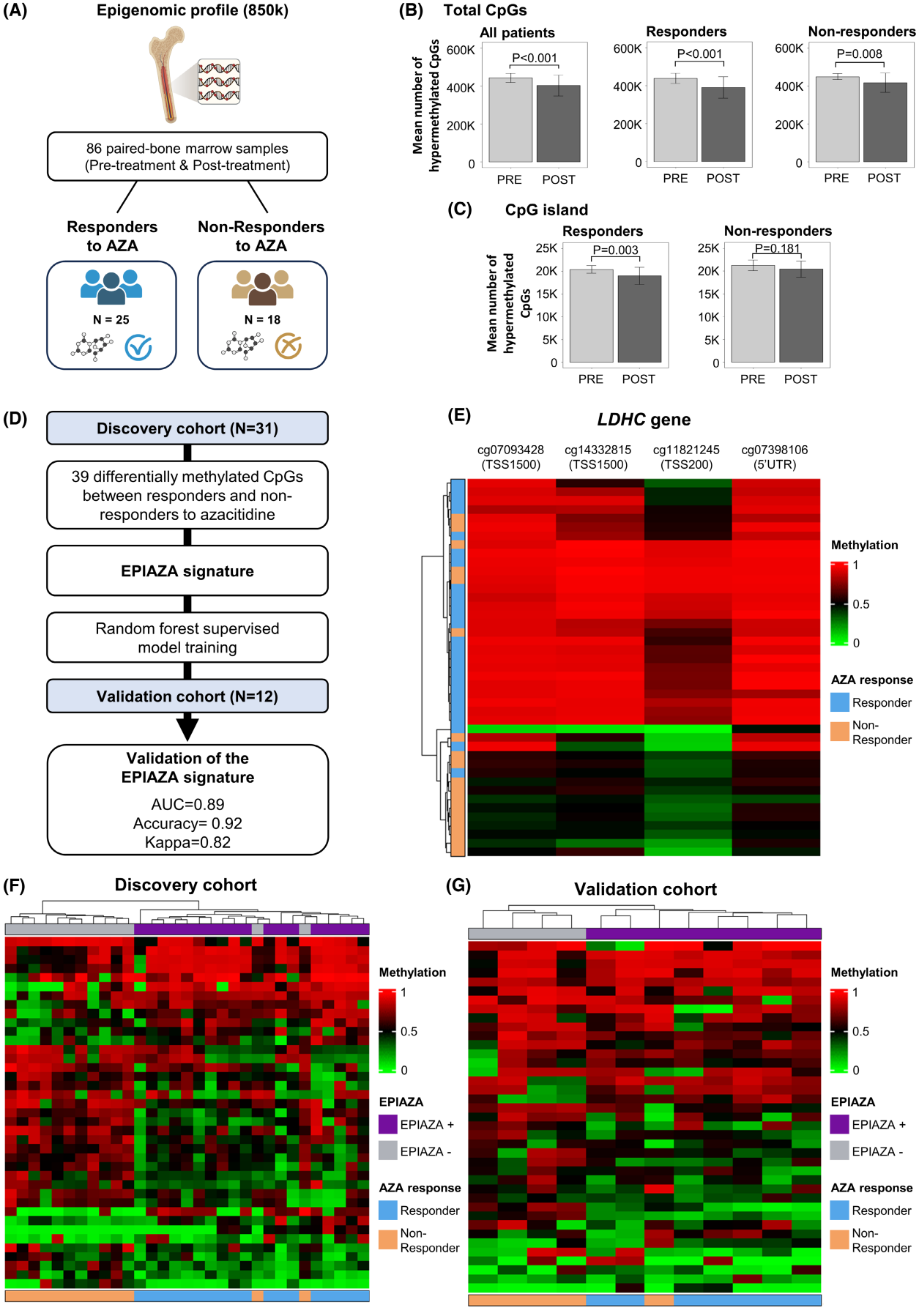
Our study includes 86 paired bone marrow (BM) samples collected prior and post-azacitidine treatment (median = 6.0 cycles [interquartile range, 4.5–6]) from 43 patients with MDS. For those few cases with less than four azacitidine cycles at evaluation response ($n = 6$), it was confirmed that the duration of response was prolonged overtime (median = 10 months). Table 1 describes the clinicopathological patient characteristics. Additional information regarding karyotypes and mutations at diagnosis is shown in Table S1. Clinical response was defined following the International Working Group response criteria in patients with MDS (IWG-MDS), as detailed in Data S1 with the pertinent references. DNA methylation profiles were generated using the Infinium HumanMethylationEPIC BeadChip microarray (EPIC/850k) (Figure 1A), as previously described.[5] Raw files (IDATs) underwent quality control, normalization and filtering to obtain the methylation scores (β- and M-values), as previously reported.[5] The global methylation content was assessed by calculating the number of hypermethylated sites. Differentially methylated CpG positions (DMPs) were identified by calculating mean β-value differences between groups and fitting a linear regression model using limma, with batch effect adjustment as a covariate. The classification model was trained by the DMPs using a random forest algorithm. Model performance was evaluated by the receiver operating characteristic (ROC) curve. For clinicopathological variables, p-values were calculated using Fisher's exact test or chi-squared test for dichotomous or categorical, respectively. For the global methylation analyses, the comparison between groups was performed with the two-sided Mann–Whitney Wilcoxon test. Statistical significance was considered if p-value <0.05; or adjusted p-value <0.05 when multiple testing was performed. Further details are available in Data S1.

RESULTS

We first determined the global DNA methylation content by calculating the number of CpG sites hypermethylated (β-value >0.66) in each patient and each condition (pre- and post-treatment). We observed, as expected, that the HMA agent induced an overall reduction of DNA methylation in post-treatment samples in comparison to the pretreatment group, evidenced by the significant decrease in the mean number of hypermethylated CpGs (Figure 1B, left). The diminished DNA methylation was observed for all genomic loci classified by CpG density and context, such as CpG-rich areas (CpG islands), neighbouring CpG island loci (CpG shelves and shores) and CpG-poor regions (open sea) (Figure S1). Interestingly, the hypomethylating effect was overall more significant in the group of HMA responders (p-value <0.001) than in non-responders (p-value = 0.008) (Figure 1B, middle and right). Even more important, the HMA responder patients showed a significant reduction of hypermethylated sites located at CpG islands (p-value = 0.003) that was not observed in the patients with MDS refractory to the azacitidine treatment (p-value =0.181) (Figure 1C). For the other CpG sites, we did not observe differences between HMA responders and non-responders, both groups showing significant hypomethylation events upon the use of azacitidine (p-value <0.05) (Figure S1). These findings could relate to the known functional reactivation of tumour suppressor genes associated with hypomethylation events at CpG islands,[6] a phenomenon that our data suggest can be invoked in the patients with MDS that respond to the epigenetic therapy. For individual CpG sites, we identified 14 loci that were uniquely hypomethylated in the post-HMA treatment samples of responder patients (Table S2). Strikingly, one of the genes associated with the identified CpG sites, phosphoinositide-phospholipase C beta1 (*PLCB1*), has been previously described as reactivated upon DNA hypomethylation in patients with MDS that responded to HMA treatment,[7,8] further validating our experimental approach.

The above comparison between the DNA methylation profiles of patients with MDS before and after azacitidine treatment according to the clinical response provides relevant clues about the further course of disease upon the use of HMAs, but considering the utility in the clinical setting, it would be optimal to decipher DNA methylation profiles that could be associated with the response taking into account only the pretreatment sample. Thus, to achieve this goal, we also conducted a differential DNA methylation analysis in HMA responders versus non-responders exclusively focusing on the BM samples prior to the azacitidine therapy ($n = 43$). These samples were divided into a discovery ($n = 31$) and validation ($n = 12$) cohorts (Table 1). To avoid any statistical bias, we confirmed that there were no significant differences between the two cohorts with respect to the clinicopathological variants, such

FIGURE 1 Impact of azacitidine on global DNA methylation patterns and definition of EPIAZA signature in patients with MDS. (A) Study design and workflow. (B, C) Bar plots representing the mean number of total hypermethylated CpG sites (B) and CpG island (C) in pretreatment (PRE) and post-treatment (POST) samples. CpG sites were categorized as hypermethylated when β-value >0.66. Two-sided Mann–Whitney–Wilcoxon test was performed. (D) Scheme of the definition of EPIAZA signature in the discovery and validation cohorts. (E) Heatmap representing the methylation β-values of four CpGs differentially methylated in responders versus non-responders, located at the 5′ regulatory region of the *LDHC* gene. CpG location is denoted below the CpG ID. The annotation bar indicates whether the sample is from a responder or non-responder patient. (F, G) Heatmaps representing the EPIAZA signature in the discovery (F) and validation (G) cohorts. Annotation bars indicate whether the sample is classified as EPIAZA+ or EPIAZA− and is from a responder or non-responder patient. Methylation β-values range from 0 (green) to 1 (red).



as age, gender, MDS WHO 2017 subtypes, BM blast count at diagnosis, IPSS-R cytogenetic risk, IPSS-R risk group and percentage of azacitidine responders (Table 1). The overall study design and pipeline are illustrated in Figure 1D. The analysis of the discovery cohort unveiled 39 CpGs with significant differences in mean methylation levels between responders and non-responders (mean delta β-value >|0.2| and adjusted p-value <0.05) (Table S3). Remarkedly, two of the genes linked to the identified CpG sites (*SLC35D2* and *SLC22B5*) belong to the SLC superfamily of solute carriers that have been previously associated with azacitidine response[9,10]; reinforcing our methodological strategy. Next, we further investigated the gene with the highest number of DMPs in responders versus non-responders (4 CpGs), the lactate dehydrogenase C (*LDHC*) gene, which was hypermethylated in HMA responders (Table S3). The methylation status of those four CpGs was enough to classify patients according to azacitidine response in pretreatment samples with a hierarchical clustering analysis (Fisher's exact test, p-value <0.001) (Figure 1E). To assess the functional role of *LDHC* hypermethylation in gene regulation, we took advantage of a panel of 23 AML cell lines[11] and found that the occurrence of hypermethylation at the 5′ regulatory region was linked to gene transcription downregulation (Spearman correlation coefficient $r = -0.548$; p-value = 0.008), in agreement with previous findings.[12] Interestingly, the upregulation of LDHC is associated with enhanced response to anti-PD-1 therapy[13]; thus, it is tempting to propose that the transcriptional reactivation of this gene by the demethylating agent might foster an immune response that could potentially improve response in patients with MDS.

We next selected the 39 methylation sites associated with HMA response to train a random forest algorithm with 10-fold cross-validation repeated three times (final mtry =2) to obtain a DNA methylation signature, hereafter termed EPIAZA. A hierarchical clustering analysis using the EPIAZA signature classified patients with MDS according to clinical response to azacitidine (Fisher exact test, p-value <0.001) (Figure 1F). Having demonstrated the EPIAZA signature ability to predict HMA-response in the discovery cohort, we assessed its performance in the validation cohort. The EPIAZA signature predicted response to azacitidine therapy with 91.7% accuracy (95% CI = 62%–99%; $\kappa = 0.82$), 80% sensitivity and 100% specificity in the MDS validation cohort. We further interrogated the model performance using the receiver operating characteristic curve, obtaining an area under the curve (AUC) value of 0.89 (Figure 1D). The application of the EPIAZA signature in the hierarchical clustering for the validation cohort of patients with MDS also distinguished HMA responders and non-responders (Fisher's exact test, p-value <0.001) (Figure 1G). Interestingly, EPIAZA prediction for clinical response to azacitidine was observed for both high and low blast percentages in BM: BM blasts 5%–19% (97% accuracy; 95% CI = 83%–99%; $\kappa = 0.93$; 92% sensitivity and 100% specificity) and BM blasts <5% (100% accuracy; 95% CI = 74%–100%; $\kappa = 1.00$; 100% sensitivity; 100% specificity). According to higher and lower risk patients with MDS by the IPSS-R classification (information available for 42 patients), 95.24% (40 of 42) of our cases were IPSS-R >3.5%, where the EPIAZA signature was also associated with HMA response (98% accuracy; 95% CI = 87%–99%; $\kappa = 0.94$; 94% sensitivity; 100% specificity). The existence of only two patients with IPSS-R <3.5 precluded any meaningful statistical analysis for this subgroup.

CONCLUSION

We have herein unveiled the DNA methylation changes that take place at a global scale in patients with MDS upon HMA treatment and found global epigenomic signatures and particular CpG sites with potential power to predict clinical response to the DNA demethylating drug. Our results indicate that, although HMA induces a profound remodelling of the DNA methylation landscape of patients with MDS, those that respond to the therapy show preferentially hypomethylating events at the CpG island regulatory genomic regions. Beyond biology, from the clinical standpoint, the analyses of the DNA methylome in the MDS sample prior to the epigenetic therapy already provides some clues about the course of the disease. In this regard, the obtained EPIAZA signature could be a useful companion to aid treatment decision by anticipating a likely response to the HMA therapy in MDS. The relevant findings from our multicentre retrospective study encourage the development of prospective assessments with higher number of patients with MDS (expanding to the effect of venetoclax combination)[14,15] to broad our understanding about the potential of DNA methylation patterns in the prediction of the clinical response to azacitidine.

AUTHOR CONTRIBUTIONS

Aleix Noguera-Castells, Ignacio Campillo-Marcos, Veronica Davalos and Carlos A. García-Prieto were involved in conceptualization, writing, and performing experiments and statistical analysis. David Valcárcel, Antonieta Molero, Laura Palomo, Norbert Gattermann, Michael Wulfert, Lorea Chaparro-González, Francesc Solé, Marta Cabezón, María J. Jiménez-Lorenzo, Blanca Xicoy, Lurdes Zamora, Alessia De Stefano, Irene Casalin, Carlo Finelli and Matilde Y. Follo were involved in data collection, haematological supervision and writing. Manel Esteller was involved in conceptualization, writing and scientific supervision.

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CONFLICT OF INTEREST STATEMENT

DV reports personal fees from BMS/Celgene, Amgen, Astellas, Agios, Grifols, Janssen, MSD, Novartis, Pfizer, Sanofi, Sobi and Takeda outside the submitted work. AM reports personal fees from Oryzon genomics and AstraZeneca; non-financial support from Novartis and Jazz pharmaceuticals outside the submitted work. LZ reports grants and personal fees from Celgene/BMS, Incyte, and Novartis outside the submitted work. CF declares research funding, advisory committees and speaker fees from Celgene BMS, advisory committees and speaker fees from Novartis and consultancy from Takeda. ME declares grants from Ferrer International and Incyte; and personal fees from Quimatryx, outside the submitted work. No disclosures were reported by the other authors.

DATA AVAILABILITY STATEMENT

For original data, please contact mesteller@carrerasresearch.org.

ETHICS STATEMENT

This study has been approved by the Research Ethics Committee of the Germans Trias i Pujol Hospital in Badalona, Catalonia, Spain (Ref. PI-21-183).

PATIENT CONSENT STATEMENT

All patients provided consent to have the clinical information reported.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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