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Impact of Epigenomic Hypermethylation at *TP53* on Allogeneic Hematopoietic Cell Transplantation Outcomes for Myelodysplastic Syndromes

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Abstract

Myelodysplastic syndromes (MDS) are a heterogeneous group of hematopoietic stem cell disorders for which allogeneic hematopoietic cell transplantation (HCT) is currently the sole curative treatment. Epigenetic lesions are considered a major pathogenetic determinant in many cancers, and in MDS, epigenetic-based interventions have emerged as life-prolonging therapies. However, the impact of epigenomic aberrations on HCT outcomes among patients with MDS are not well understood. We hypothesized that epigenomic signatures in MDS patients before undergoing HCT serve as a novel prognostic indicator of the risk of post-HCT MDS relapse. To evaluate these epigenomic signatures in MDS patients, we analyzed reduced representation bisulfite sequencing profiles in a matched case-control population of 94 patients. Among these HCT recipients, 47 patients with MDS who relapsed post-HCT (cases) were matched 1:1 to patients with MDS who did not relapse (controls). Only patients with wild-type *TP53*, RAS pathway, and *JAK2* mutations were included in this study to promote the discovery of novel factors. Cases were matched with controls based on conditioning regimen intensity, age, sex, Revised International Prognostic Scoring System, Karnofsky Performance Status, graft type, and donor type. Pre-HCT whole-blood samples from cases and matched controls were obtained from the Center for International Blood and Marrow Transplant Research repository. We comprehensively identified differentially methylated regions (DMRs) by comparing the methylation patterns among matched cases and controls. Our findings show that cases displayed

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SUPPLEMENTARY MATERIALS

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more hyper-DMRs pretransplantation compared with controls, even after adjusting for pre-HCT use of hypomethylating agents. Hyper-DMRs specific to cases were mapped to the transcription start site of 218 unique genes enriched in 5 different signaling pathways that may serve as regions of interest and factors to consider as prognostic determinants of post-HCT relapse in MDS patients. Interestingly, although patients selected for this cohort were wild-type for the *TP53* gene, cases showed significantly greater levels of methylation at *TP53* compared with controls. These findings indicate that previously identified prognostic genes for MDS, such as *TP53*, may affect disease relapse not only through genetic mutation, but also through epigenetic methylation mechanisms.

Keywords

Myelodysplastic syndromes; Hematopoietic cell; transplantation; Transplantation outcome; DNA methylation; Epigenomics; *TP53*

INTRODUCTION

Myelodysplastic syndromes (MDS) are a heterogeneous group of sporadic and typically late-onset clonal hematopoietic progenitor disorders that cause insufficient myeloid, erythroid, and thrombocyte differentiation. In addition to complications from progressive transfusion dependence, patients with MDS have an increased risk of acute myelogenous leukemia (AML) [1]. Approximately 10,000 adults are diagnosed with MDS in the United States each year [2]. The prognosis for individuals with MDS is stratified according to disease- and patient-specific risk factors. The Revised International Prognostic Scoring System algorithm is commonly used to predict overall survival, which ranges from less than 1 year to more than 8 years from diagnosis [3,4].

Current therapy for adults with MDS varies from watchful waiting for those with very low-risk disease, through growth factor support, red cell and platelet transfusion, hypomethylating agent (HMA) therapy, intensive chemotherapy, and allogeneic hematopoietic cell transplantation (allo-HCT) for high-risk or very-high-risk disease. Although HMA therapy improves survival and reduces the need for transfusions, allo-HCT remains the sole available curative-intent treatment for MDS. Previous studies have shown that genetic mutations in the *TP53*, *RAS* pathway, and *JAK2* genes strongly correlate with poor post-transplantation outcomes [5]. However, among patients with wild-type *TP53*, *RAS* pathway, and *JAK2* genes, any further explanation for poor outcomes post allo-HCT was not determined.

Epigenetic mechanisms control the spatial organization and transcriptional programming of genomes through reversible, covalent modification of DNA. MDS are characterized by epigenetic aberrations, specifically DNA hypermethylation. Hypermethylation results in transcriptional repression at proximal genes. DNA methylation is highly correlated with chronological age and clonal hematopoiesis and is also one of the most critical risk factors for MDS progression to AML [6–10]. The striking effect of methylation provides a rationale for the use of HMA therapy for these diseases.

We hypothesize that epigenomic signatures in patients with MDS before undergoing HCT serve as a novel prognostic indicator of post-HCT MDS relapse risk. To evaluate these epigenomic signatures in MDS patients, we used reduced representation bisulfite sequencing to identify genome-wide methylation profiles. We leveraged this whole-genome methylation data in a matched case-control population from the Center for International Blood and Marrow Transplant Research (CIBMTR) for patients undergoing HCT with wild-type *TP53*, RAS pathway, and *JAK2* genes [5] to determine novel prognostic factors in post-HCT MDS relapse risk in the epigenomic arena where previous genomic analyses were unable to further risk-stratify patients.

METHODS

Cohort Selection

To investigate previously undiscovered pretransplantation determinants of post allo-HCT MDS relapse, we selected a group of 94 MDS patients who were previously identified as wild-type for *TP53*, RAS pathway, and *JAK2* mutations [5]. In this previous study, confirmation of the wild-type status of *TP53*, RAS pathway, and *JAK2* genes for this cohort was performed through a targeted sequencing approach. The population was stratified by disease relapse status (cases versus controls) and matched on patient age ± 5 years, conditioning regimen, Revised International Prognostic Scoring System, Karnofsky Performance Status, donor and graft type, and duration of post-HCT follow-up. Characteristics of the patient cohort are presented in Table 1, where 47 patients with MDS who relapsed post-HCT (cases) were matched 1:1 to MDS patients who did not relapse (controls). In the control group, no clinical evidence of disease relapse was reported within 5 years after allo-HCT. The Pearson chi-square test was used for comparing discrete variables, and the Kruskal-Wallis test was used for comparing continuous variables. The study was approved by the National Marrow Donor Program's Institutional Review Board and conducted in accordance with the Declaration of Helsinki.

DNA Collection, Extraction, and Methylation Sequencing

Peripheral whole blood samples were collected before administration of the preparative conditioning regimen for allo-HCT and processed centrally at the CIBMTR repository using standard operating procedures. Genomic DNA was extracted from these whole blood samples, and methylation sequencing was performed. DNA (150 to 300 ng) was digested at CpG motifs with MspI (10 U, R0106L; New England BioLabs, Ipswich, MA). Digested fragments underwent end repair and A-tailing with Klenow fragment polymerase (5 U, M0212S; New England BioLabs) and overnight ligation with TruSeq adapters (Illumina, San Diego, CA) containing next-generation barcode sequences. DNA cleanup and fragment size selection were completed using Agencourt AMPure beads (Beckman Coulter, Indianapolis, IN) at 2 \times beads to sample volume. Samples were then processed twice for bisulfite conversion using the EpiTect Bisulfite conversion kit (Qiagen, Hilden, Germany). Once DNA cleanup was completed, relative abundance (SYBR Green qPCR; Bio-Rad, Hercules, CA) and PCR amplification of the products (Pfu Turbo; Agilent, Wilmington, DE) were performed. Libraries were pooled in equimolar ratios and underwent size selection using 1.2 \times and 0.7 \times Agencourt AMPure beads (Beckman Coulter) to enrich for 150-

to 500-bp products. Final assessment and quantification of the reduced representation bisulfite sequencing libraries was completed with qPCR, fluorescence measurements, and a Bioanalyzer 2100 DNA high-sensitivity assay (Agilent). Next-generation sequencing was completed on an Illumina HiSeq 2500 DNA sequencer. Paired-end sequencing of 125-bp reads was generated at >10× sequencing depth.

Analytical Pipeline

The Bismark bioinformatics toolkit [11] was used to trim sequencing adaptors and align sequencing reads to the human reference genome for calling methylation profiles. After quality control, low-coverage CpG sites (defined as <10 methylation calls) were excluded during the identification of differentially methylated regions (DMRs). A Python application, methylPrep, was built to aggregate shared CpG sites into groups. The MethylKit R package [12] was applied to identify DMRs (50-bp intervals), with at least a 25% difference in methylation calls [13,14]. DMRs are defined as regions with significantly differential methylation between a case and its matched control. DMR annotation was processed to identify the genomic regional distribution of DMRs by the Genomation R package [15] and also to identify genes that potentially could be impacted by DMRs. Subsequently, these genes were used for pathway enrichment analysis ($P < .05$) using Reactome pathway analysis [16].

RESULTS

Cases Have More Hypermethylated Regions Than Controls

Variation in genome-wide methylation was defined by first delineating the genome into regions of 50-bp intervals and then filtering for regions with at least a 25% difference in methylation calls between cases and controls. A higher number of hypermethylated regions than hypomethylated regions ($P = 2.86E-04$) was observed comparing each relapsed case with the respective matched nonrelapsed control. There were many thousands of hyper-DMRs and hypo-DMRs, and the dominant pattern was hypermethylation in cases (Figure 1A).

Promoter Regions are Enriched for Hyper-DMRs among Cases

Cases displayed significantly greater levels of hypermethylation compared with controls in the promoter, exon, intron, and intergenic regions genome-wide (Figure 1B). We noted that a large proportion (19%) of hypermethylated regions resided in the promoter region (Figure 1B), a relatively small portion of the genome. These hypermethylated regions mapped to the transcription start site (TSS) of 218 unique genes (Supplementary Table S1), including *TP53*, *RIPK1*, *TNSF10*, and *CEBPA*. These genes were overrepresented ($P < .05$) in MDS/AML-related signaling pathways, including TP53, RAS, MAPK, TGF, and TNF pathways (Supplementary Table S2).

Preferential Hypermethylation at the TSS of TP53 in Pretransplantation Samples of Cases

Because *TP53* plays such a large role in MDS outcomes, we explored both hyper-DMRs and hypo-DMRs specifically in the *TP53* gene region. We observed 7 hyper-DMRs and 4 hypo-DMRs in the case versus control direction near *TP53*. More than one-half of

these hyper-DMRs were located in the TSS, and one was located in exon 2 (Figure 2A). The DMRs shown here represent significant DMRs detected in pretransplantation samples derived from cases and controls, where hyper-DMRs displayed higher methylation in the cases than in the controls and vice versa for hypo-DMRs. These bars indicate mean values of differentially methylated cytosine regions across cases and controls and do not represent individual samples.

Methylation Differences in Cases versus Controls Are Agnostic of HMA Treatment

Because many patients receive HMA treatment before transplantation, and to account for potential HMA treatment bias, we specifically examined methylation levels in the subset of patients treated with HMA. Among HMA-treated patients, cases still showed more hyper-DMRs than hypo-DMRs ($P = 2.45E-02$). For all patients as well as HMA-treated patients (Figure 2B,C), more methylated CpG sites were detected at gene features near *TP53* in cases versus controls ($P < .05$). Thus, the methylations differences detected in cases versus controls before transplantation were agnostic of HMA treatment.

DISCUSSION

In a nested case-control study, we analyzed the impact of novel epigenomic risk factors on post-allo-HCT relapse in a cohort of MDS patients who were wild-type for *TP53*, RAS pathway, and *JAK2* gene mutations and were 1:1 matched on established clinical prognostic factors. Other studies have shown that global hypermethylation in MDS patients may reduce the expression of tumor suppressors [17,18]. Our results identify differentially methylated regions between cases and controls, especially hypermethylation in promoter regions that may be involved in down-regulation of gene expression. These promoter DMRs may serve as novel prognostic factors of allo-HCT outcomes.

Despite the exclusion of patients with genetic mutations in *TP53*, RAS pathway, and *JAK2* genes, our results show that these genes and pathways still may play a critical role through epigenomic/methylation mechanisms. A significant number of DMRs were detected in these regions between cases and controls. Specifically, a higher number of hypermethylated cytosines were detected at *TP53* in cases versus controls. These data suggest that epigenetic factors may provide new insight into determinants of allo-HCT outcomes, even among patients with wild-type *TP53* genes. Here we present a model in which either genetic mutation or hypermethylation in key genes can lead to MDS disease relapse after allo-HCT (Figure 3).

The status of DNA methylation in many genes is associated with survival and therapy response in MDS patients in whom decreases in methylation are predictive of better outcomes [19,20]. Treatment of patients with DNA methyltransferase inhibitors has long been the standard of care to improve survival for most MDS patients [21]. Although we show that the presence or absence of treatment with HMA therapy did not change the key differences in methylation seen between our cases and controls, we did not have details of specific HMA therapy dose or duration which may oversimplify the potential impact of HMA on epigenetic changes. Regarding the pathology of MDS and progression to AML or response to therapies, changes in methylation may activate or inhibit expression of key

genes, block or inactivate functional pathways, or release holds to stimulate growth and differentiation. The identification and elucidation of the role of specific potential prognostic epigenetic mutations require further exploration of global methylation patterns and changes to distinguish distinct MDS subtypes [9].

With respect to the potential mode of action for methylation regulation, previous studies have observed that global hypermethylation may reduce the expression of tumor suppressors, such as TP53 and FOXP3 [22], as well as genes that regulate leukocyte telomere length, such as telomere reverse transcriptase (*TERT*). Indeed, patients with shorter telomeres experience higher post-allo-HCT treatment-related mortality (TRM) [23,24]. Acquired mutations in genes for epigenetic regulators are also implicated in the early stages of MDS evolution. Examples of these mutations and their relative prevalence in MDS include mutations in the genes encoding ASXL1 (20% of MDS) [25], TET methylcytosine dioxygenase 2 (*TET2*; 15% to 35%) [26,27], and members of the histone-lysine demethylase gene family (*KDM5A*, *KDM6A*, and *KDM2B*; 1% to 4%) [28] and others with differential methylation profiles observed in this study.

Further investigation will add to the validation of the impact and mode of action of these distinct methylation signatures and global methylation patterns in MDS associated with HCT outcomes. In previous MDS studies, high concordance has been found in results from molecular assays between peripheral blood and bone marrow [29,30]. Assessment of overall whole-genome profiles at additional time intervals and disease or transplantation stages would help explore linkages to causal effects. The use of other omics methodologies will also help address the question of whether additional genomic mutations and/or other omics signatures may be prognostic of allo-HCT outcomes in MDS. Because some major methylation effects are known to occur at the step of transcription regulation, hypothesized effects on protein changes could be evaluated through proteomics explorations. These will help shed light on potential mechanisms of action downstream of differences detected in methylation profiles.

These findings provide potential new insights into MDS relapse after allogeneic HCT. Relapse associations with patient methylation profiles before HCT provides potential targets for personalized therapeutic interventions. Especially when narrowed to detection at specific prognostic genes, such as *TP53*, such testing could become feasible for consideration in realtime therapeutic decision making for MDS patients when multiple avenues forward exist. The addition of methylation testing to the amplification of the marker *TP53* gene does not add significant cost to targeted panel testing of DNA methylation sites or genetic variant markers, especially compared with whole-epigenome or genome-wide assays. By adding the striking *TP53* methylation marker to a prognostic MDS patient transplantation outcome panel test composed of even a dozen of the top prognostic markers from previous studies, the potential algorithm could be improved over assays and technologies that seek to measure hundreds to thousands of markers. Further investigation through validation in larger populations and evaluation of the individual feature contributions of omics marker associations with MDS patient transplantation outcomes will lead to the development of a predictive algorithm with high translational utility in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of interest statement:

Dr. Nazha declares Jazz Pharmaceuticals for research support, Incyte and Novartis for Speakers Bureau, MEI for the Data Monitoring Committee, Karyopharma, Tolero, Abbvie and Daiichi Sankyo for Advisory Board or consulting. Dr. Carlson is a member of the NMDP IRB. All other authors have no interest conflicts to declare for this manuscript.

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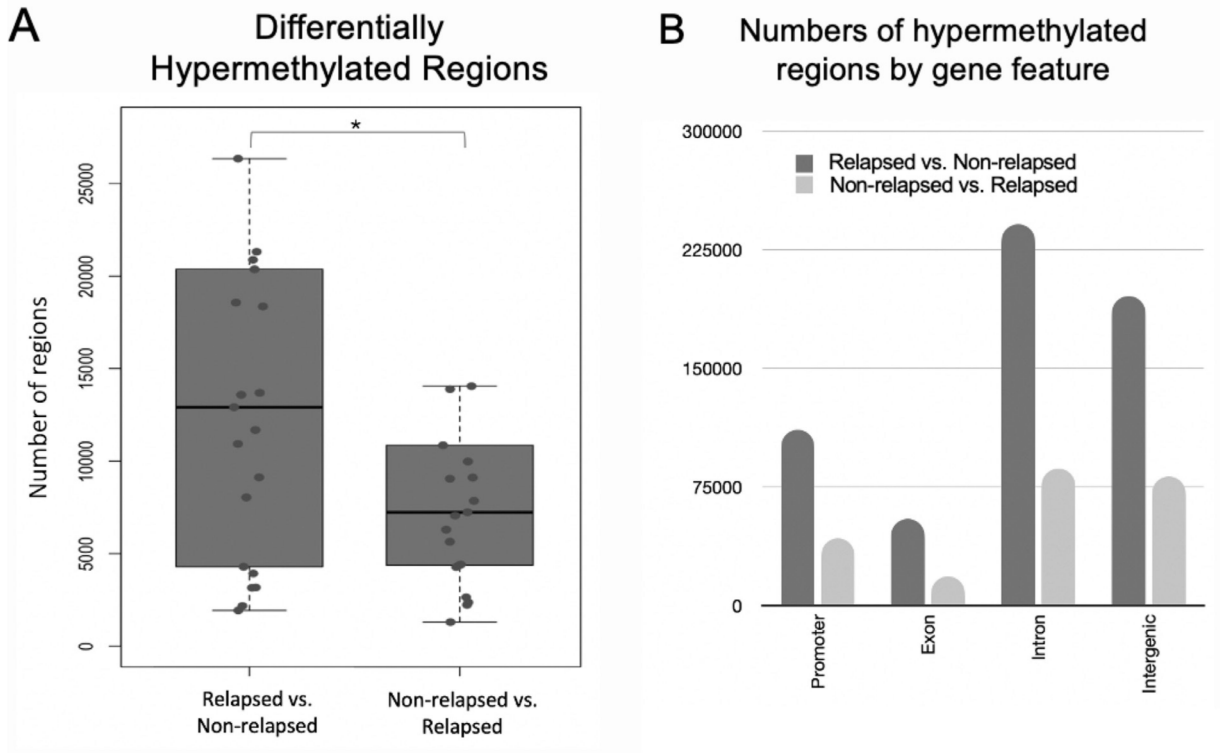


Figure 1. Distribution of DMRs in cases (relapsed) and controls (nonrelapsed). **(A)** There were more regions of hypermethylation in cases compared with controls than in the reverse direction. **(B)** Total number of differentially methylated cytosines by genomic features, with the ratio of hypermethylation in cases versus controls in dark gray and vice versa in light yellow. DMRs were enriched in promoters.

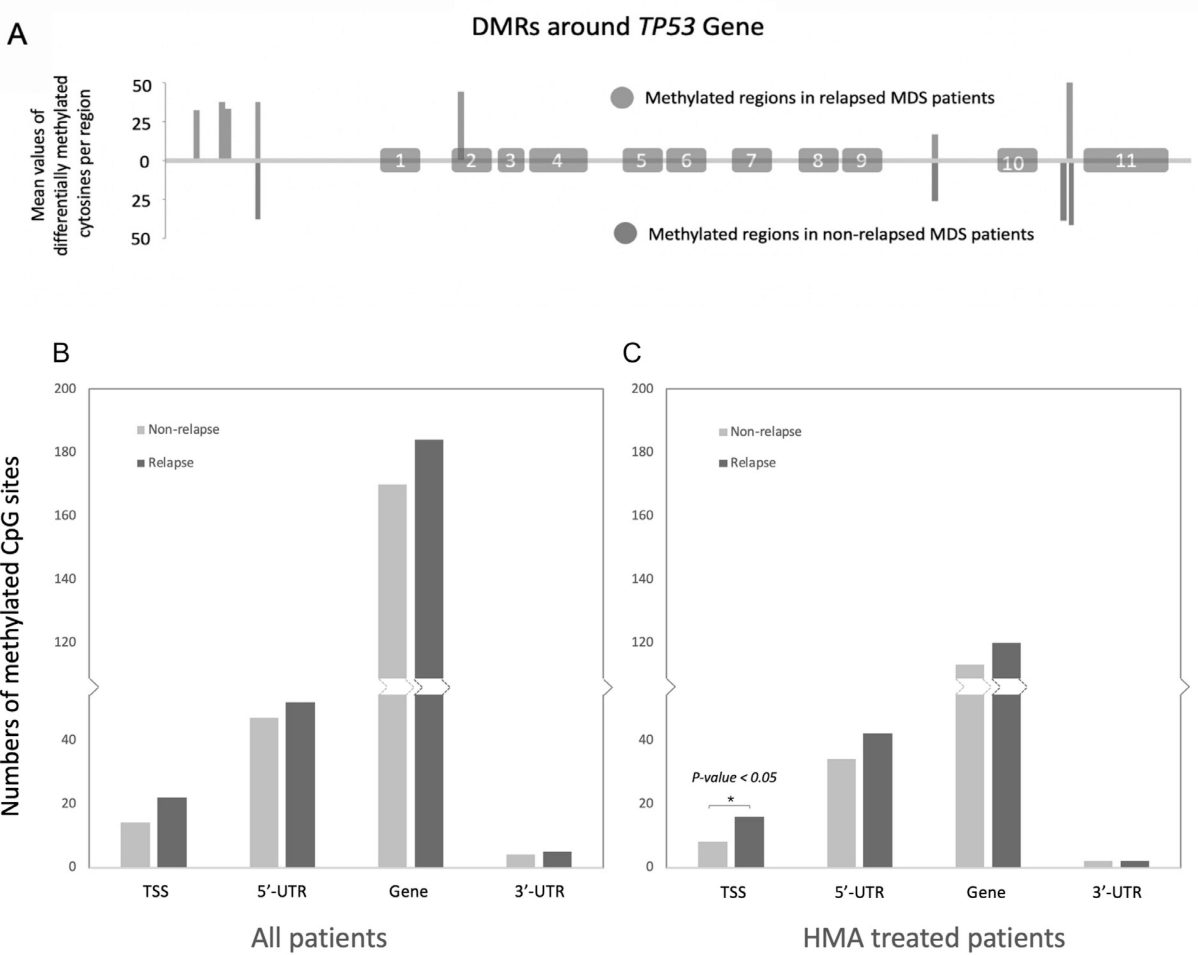


Figure 2.

Significantly more hypermethylated cytosines compared with hypomethylated cytosines were detected in the transcription start site of *TP53* in cases (relapsed) versus controls (nonrelapsed). (A) DMRs near *TP53* with exons numbered where hypermethylated regions are shown above the horizontal line, and hypomethylated regions appear below the line. (B) Differentially methylated regions by genomic feature near *TP53* (hypermethylated shown in dark gray, hypomethylated in light gray). (C) As in (B) but considering only patients treated with HMA.

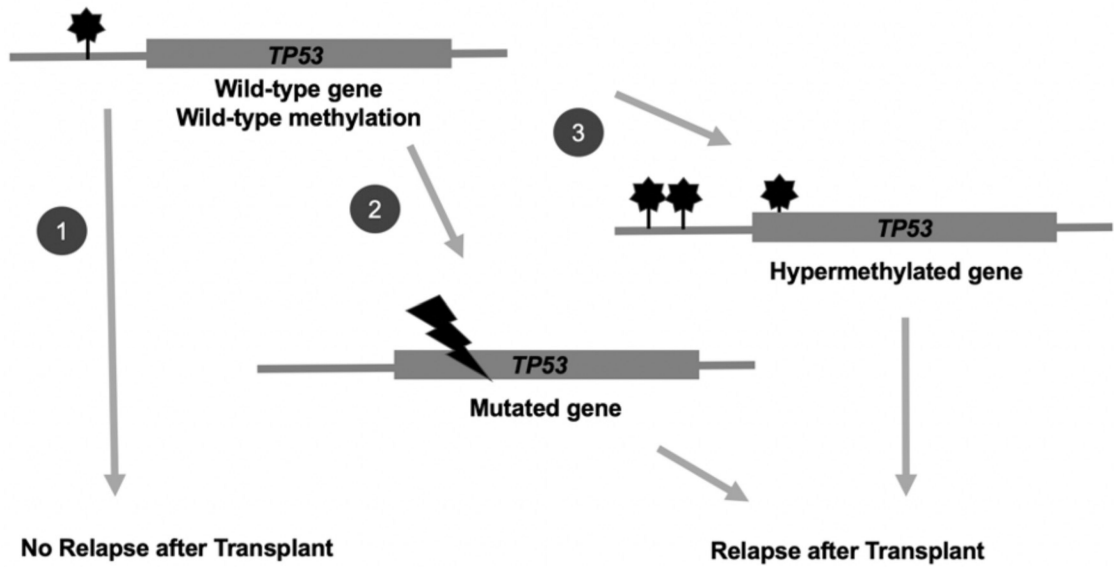


Figure 3.

Transplantation outcomes in MDS patients may be determined by the presence of genetic mutations or epigenetic hypermethylation in key prognostic genes, such as *TP53*. In scenario (1), patients with the wild-type *TP53* gene at wild-type methylation levels pretransplantation do not experience relapse post-transplantation. However, those patients with (2) genetic mutations at *TP53* or (3) hypermethylation at *TP53* pretransplantation are at increased risk of MDS disease relapse post-transplantation.

Table 1

Characteristics of 94 MDS Patients without *TP53*/RAS Pathway/*JAK2* Gene Mutations and Split in Cases versus Controls *

| Variable | Controls | Cases |
|---------------------------------------|------------|------------|
| Number of patients | 47 | 47 |
| IPSS-R score, n (%) | | |
| Very low | 4 (9) | 4 (9) |
| Low | 14 (30) | 14 (30) |
| Intermediate | 23 (49) | 23 (49) |
| High | 5 (11) | 5 (11) |
| Very high | 1 (2) | 1 (2) |
| Recipient age at transplantation, yr | | |
| 50–59, n (%) | 9 (19) | 9 (19) |
| 60+, n (%) | 38 (81) | 38 (81) |
| Median (range) | 64 (53–70) | 64 (53–70) |
| Recipient race, n (%) | | |
| Caucasian | 43 (93) | 46 (98) |
| Recipient sex, n (%) | | |
| Male | 29 (62) | 30 (64) |
| Karnofsky Performance Status, n (%) | | |
| 90–100 | 31 (66) | 31 (66) |
| Donor product source, n (%) | | |
| HLA-identical sibling and PBSCs | 3 (6) | 3 (6) |
| 8/8 URD and PBSCs | 44 (94) | 44 (94) |
| Conditioning regimen intensity, n (%) | | |
| Myeloablative | 16 (34) | 16 (34) |
| Reduced intensity | 31 (66) | 31 (66) |
| Year of transplantation, n (%) | | |
| 2004–2005 | 1 (2) | 0 |
| 2006–2007 | 3 (6) | 5 (11) |
| 2008–2009 | 7 (15) | 9 (19) |
| 2010–2011 | 11 (23) | 3 (6) |
| 2012–2013 | 25 (53) | 30 (64) |
| Pre-HCT HMA and chemotherapy, n (%) | | |
| HMA only | 27 (57) | 34 (72) |
| Chemotherapy only | 1 (2) | 1 (2) |
| HMA and chemotherapy | 4 (9) | 1 (2) |
| None | 15 (32) | 11 (23) |

IPSS-R indicates Revised International Prognostic Scoring System; PBSCs, peripheral blood stem cells; URD, unrelated donor.

* Cases and controls were matched on follow-up from diagnosis to relapse.

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