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***TP53* and *IDH2* somatic mutations are associated with inferior overall survival following allogeneic hematopoietic cell transplantation for myelodysplastic syndrome**

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Abstract

Next generation sequencing has identified somatic mutations that are prognostic of cancer. We evaluated the incidence and prognostic significance of somatic mutations in 89 MDS patients who received an allogeneic hematopoietic cell transplant. Next generation sequencing was performed on paraffin embedded bone marrow which was obtained at a median of 31 days prior to initiating the preparative regimen. The 3 most common subtypes of MDS were RAEB-1 (35%), RAEB-2 (29%) and RCMD (18%). Most patients (n=91%) received a myeloablative regimen of fludarabine plus intravenous busulfan. Somatic mutations (>0) were identified in 39 (44%) of analyzed samples. The six most commonly identified gene mutations were *ASXL1* (8%), *DNMT3A* (8%), *RUNX1* (7%), *KRAS* (6%), *IDH2* (4%), and *TP53* (4%). The low incidence of mutations in our study sample may be explained by tissue source and stringent variant calling methodology. Moreover, we speculate that the low incidence of mutations may, perhaps, be also explained by prior azacitidine treatment in 82% of cases. Multivariate analysis identified *TP53* (HR=3.82 (95%CI=1.12, 13.09, p=0.03) and *IDH2* mutations (HR=4.74 (95%CI=1.33,16.91), p=0.02) as predictors of inferior 3-year overall survival. This study furthers implementation of clinical genomics in MDS and identifies *TP53* and *IDH2* as targets for pre- or post-transplant therapy.

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Keywords

Myelodysplastic syndrome; somatic mutations; allogeneic hematopoietic cell transplantation; next generation sequencing

Introduction

Despite growing understanding of the genetic and molecular basis of myelodysplastic syndromes (MDS) as well as availability of epigenetic therapy, the disease remains incurable unless eligible patients are offered an allogeneic hematopoietic cell transplant (allo-HCT).^{1–6} Nevertheless, only approximately half of the cases who undergo the procedure achieve durable remissions.^{5, 6} Significant morbidity and mortality occurs despite efforts to optimize patient selection using disease-specific HCT comorbidity indices.^{7, 8}

No randomized data exist which compares allo-HCT against non-transplant therapies in patients with MDS. Presently, the decision to offer an allo-HCT is based on published decision models which have demonstrated a significant benefit favoring offering an allo-HCT, earlier in the disease course, for patients with intermediate- or higher-risk MDS whether allografted from histocompatible siblings or unrelated donors.^{9, 10} Published retrospective single institution or registry studies have also supported the benefit of an allo-HCT to eligible patients with MDS.^{11–14} Various clinical models have been developed aimed at improving outcomes in patients undergoing an allo-HCT for MDS by identifying patient's comorbidities^{7, 15} and clinical characteristics of the disease.¹⁶ These models, however, are unable to predict outcomes in individual cases.

Availability of next generation sequencing (NGS) has helped identify somatic mutations capable of prognosticating outcomes of patients with MDS and acute myeloid leukemia.^{1, 17, 18} Bejar et al. evaluated, retrospectively, 87 subjects who underwent an allo-HCT for MDS, median age 58 (19–73) years, and identified presence of at least one somatic mutations in 92% of cases.¹⁹ In their series, mutated *ASXL1* was the most frequently observed mutation (29%).¹⁹ Moreover, mutations in *TP53*, *TET2*, and *DNMT3A* were associated with an inferior overall survival (OS) following allo-HCT; and presence of mutated *TP53* was described as the most significant predictor of mortality after transplantation with a short median survival of 4.6 months.¹⁹ This study highlights the importance of identifying strong predictors of adverse outcomes in patients with MDS undergoing allo-HCT in order to develop therapeutic strategies aiming at improving outcomes.¹⁹ Integrating NGS as a prognostic tool represents a step in the right direction aiming at moving MDS therapeutics to a more personalized approach.

Patients and methods

Patient and treatment characteristics

Patients were considered eligible for inclusion in this study if they were ≥ 18 years of age, received an allo-HCT for MDS between 01/2005 and 06/2012, and had available formalin-fixed paraffin embedded (FFPE) bone marrow samples from the pre-transplant work up which was performed within approximately 30 days from initiation of the preparative

regimen. All samples were collected under a protocol approved by the Institutional Review Board of the University of South Florida. This study was conducted in accordance with the Declaration of Helsinki.

There were no limitations pertaining to use of a particular cell source (bone marrow (BM) or G-CSF mobilized peripheral blood stem cells (PBSC) or cord blood), donor source (related or unrelated) or HLA-matching status. HLA-matching was defined as 8/8 (class I: A, B, C; class II: DRB1). Moreover, there were no restrictions relating to the intensity of the preparative regimen used, whether myeloablative (MAC) or reduced-intensity (RIC) conditioning, or to a particular regimen for prophylaxis against graft-versus-host disease (GVHD). Acute graft-versus-host disease (GVHD) was graded according to 1994 consensus criteria²⁰ and chronic GVHD by using the National Institutes of Health consensus development published in 2005.²¹

Methods for DNA sequencing and mutational analysis

DNA was isolated from three 20 micron FFPE sections using the Qiagen DNA isolation kit supplemented with Uracil- DNA glycosylase to remove sections of nucleotides with FFPE related artifacts. Subsequently, 100ng of DNA was used to generate NGS libraries that were amplicon enriched for all coding regions of: *ASXL1*, *TET2*, *SETBP1*, *IDH1*, *IDH2*, *EZH2*, *DNMT3A*, *TP53*, *SRSF2*, *U2AF1*, *ZRSF2*, *SF3B1*, *FLT3*, *NRAS*, *KRAS*, *CBL*, *KIT*, *JAK2*, *MPL*, *ABL1*, *RUNX1*, *ETV6*, *WT1*, *CEBPA*, *MLL*, and *NPM1*. Barcoding and pooled samples were sequenced on the Illumina platform with an average read depth of 1500x. The Qiagen GeneRead software suite was used for alignment and variant calling. Frameshift, nonsense, and missense variants that were not present in germline databases (exome variant server and 1000 genome project) at greater than 1% frequency and those that were predicted to be functionally significant by SIFT and Polyphen were deemed true mutations. The cutoff for variant allele frequency was set at 10% to differentiate from FFPE artifact.

Statistical methods

The baseline patient characteristics were summarized using descriptive statistics including median, standard deviation and range for continuous measures and proportions and frequencies for categorical measures. Probabilities of 3-year progression-free survival (PFS) and OS were calculated from the date of stem cell infusion using the Kaplan Meier method and the difference for various independent categorical variables was assessed using the Log-Rank test. Statistical significance was set at <0.05 for all comparisons. Multivariate analysis for PFS and OS was performed by means of the Cox proportional hazards (PH) regression model. The final multivariable model was selected by allowing variables with p<0.1 to remain in the model and adjusting for percentage of BM myeloblasts, presence of >0 mutation, and R-IPSS. Cumulative incidences of acute and chronic GVHD, relapse/progression (CIR/P) and non-relapse mortality (NRM) were generated and compared using the method of Gray. A two-sided p-value of <0.05 was the defined cutoff for statistical significance. Statistical analysis was performed using SPSS version 22 (IBM Analytics, Armonk, NY, USA).

Results

Patient, disease, and transplant related characteristics

Initially, we identified 139 patients who received an allo-HCT during the specified time period. However, 38 were excluded for inability to isolate DNA due to poor quality of FFPE bone marrow samples and 12 due to diagnoses other than MDS (AML=4, CMML=8).

Accordingly, 89 patients (male=52%), median age 58 (22–74) years, were included in this cohort. The three most common MDS types at time of diagnosis, as defined by 2008 World Health Organization (WHO) criteria, were refractory anemia with excess blasts 1 (RAEB-1, 35%), RAEB-2 (29%) and refractory cytopenia with multilineage dysplasia (RCMD, 18%) (Table 1). A large majority of patients had received azacitidine prior to allo-HCT (82%) and 26% of them had achieved at least a partial response (PR) (complete remission (CR)=9%, CR marrow=4%, PR=13%).

At time of allo-HCT, over one-half of cases (51%) had intermediate, high, or very-high risk disease according to the Revised International Prognostic Scoring System for Myelodysplastic Syndrome (R-IPSS). The majority of patients received G-CSF mobilized PBSC (96%) mostly from unrelated donors (matched-unrelated (MUD)=48%, mismatched-unrelated (MMUD)=15%, cord blood=3%) (Table 1). The largest majority of patients (n=93, 92%) received a myeloablative conditioning (MAC) regimen consisting of fludarabine plus intravenous busulfan; and the cumulative busulfan area under the curve (AUC) ranged from 3500–7500 mol·min/L (3500 (n=15), 5300 (n=59), 6000 (n=4), and 7500 (n=3). Tacrolimus plus methotrexate was the most commonly used regimen for GVHD prophylaxis (64%). These and other patient-, donor-, and disease-related characteristics are summarized in Table 1.

Mutational analysis

Incidence—Bone marrow samples for mutational analysis were obtained within a median of 31 (9–76) days prior to initiation of the preparative regimen. Somatic mutations (>0) at the time of allo-HCT were identified in 39 (44%) of analyzed BM samples. The most common mutations in decreasing order frequency were: *ASXL1* (8%), *DNMT3A* (8%), *RUNX1* (7%), *KRAS* (6%), *IDH2* (4%), *TP53* (4%), *SRSF2* (3%), *SF3B1* (3%), *EZH2* (3%), *TET2* (2%), *WT1* (2%), *CBL* (1%), *MLL* (1%), *MPL* (1%), *KIT* (1%), *ZRSF2* (1%), and *U2AF1* (1%). There was no significant correlation between age and presence of >0 mutation (age <60=37% vs. age ≥60=45%, Spearman Correlation $p=0.88$).

Survival—The median follow-up for surviving patients was 49 (12–100) months. The median survival for all patients was 29 months. Patients who harbored a *TP53* or *IDH2* mutations had median survivals of 6.1 months and 8.4 months, respectively.

3-year PFS: The 3-year PFS for all patients was 20% (95%CI=13–30%). No difference in 3-year PFS was observed between older (≥60 years) (13% (95%CI=5–28%) vs. younger patients (25% (95%CI=15–39%), $p=0.15$).

3-year PFS was not affected by the presence of >0 mutation (>0=23% (95%CI=12–39%) vs. no mutation=18% (95%CI=9–31%), $p=0.56$), R-IPSS category high (high/very high=23%

(95%CI=12–39%) vs. very low/low/intermediate=19% (95%CI=10–33%), $p=0.65$), or presence of $\geq 5\%$ myeloblasts in pre-transplant BM ($\geq 5\%=22\%$ (95%CI=10–41%) vs. $<5\%=19\%$ (95%CI=10–31%), $p=0.69$).

3-year OS: The 3-year OS for all patients was 49% (95%CI=39–59%). No difference in 3-year OS was observed between older (≥ 60 years) (52% (95%CI=36–68%) vs. younger patients (47% (95%CI=33–61%), $p=0.60$). Moreover, patients harboring mutated *TP53* or *IDH2* had statistically significant inferior 3-year OS ((*TP53* mutated=0 vs. wild type=51% (95%CI=41–62%), $p=0.04$); 3-year OS (*IDH2* mutated=0 vs. 51% (95%CI=41–62%), $p=0.04$)) (Figures 1–2).

3-year OS was not affected by presence of >0 mutation ($>0=51\%$ (95%CI=35–66%) vs. no mutation=48% (95%CI=34–62%), $p=0.76$) (Figure 3), R-IPSS category high (high/very high=48% (95%CI=33–64%) vs. very low/low/intermediate=53% (95%CI=38–67%), $p=0.68$), or presence of $\geq 5\%$ myeloblasts in pre-transplant BM ($\geq 5\%=48\%$ (95%CI=31–65%) vs. $<5\%=50\%$ (95%CI=37–62%), $p=0.89$).

Multivariate analysis: Multivariate analysis identified presence of *IDH2* mutation as predictors of inferior 3-year PFS and OS. Presence of *TP53* mutation predicted for inferior 3-year OS but not 3-year PFS (Table 2).

Relapse/progression

The CIR/P for all patients was 26% (95%CI=18–37%) at 1 year and 47% (95%CI=38–60%) at 3 years. The 3-year CIR/P was significantly higher in patients ≥ 60 years of age (66% (95%CI=51–85%) vs. 34% (95%CI=23–51%), $p=0.0002$).

Acute and chronic GVHD and non-relapse mortality

The cumulative incidences of acute GVHD grades II–IV and grades III–IV by day 100 for the entire cohort were 80% (95% CI= 72–89%) and 20% (95% CI=13–31%), respectively. The 1-year and 3-year cumulative incidence of moderate or severe chronic GVHD for the entire cohort were 47% (95%CI= 38–59%) and 54% (95% CI= 44–65%), respectively.

The 100 day, 1 year, and 3 year cumulative incidences of NRM for the entire cohort were 7% (95%CI 3–15%), 19% (95%CI 12–29%), and 28% (20–39%), respectively.

Discussion

Presence of mutated *TP53* is associated with an inferior OS and a strong trend for worse PFS in patients with MDS undergoing an allo-HCT (Table 2). In our study, the median survival of patients harboring *TP53* mutations was only 6.1 months and the 3-year PFS and OS were 0. Lindsley et al. showed that *TP53* mutations are associated with shorter survival and a shorter time to relapse.²² These findings are comparable to a previous single-institution study which reported a median survival of 4.6 months in mutated *TP53* MDS.¹⁹ In contrast to Bejar et al. who reported an incidence of mutated *TP53* of 21%¹⁹, in ours it was only 4%. Our study identified a relatively low frequency of mutations (>0) (44% vs. 92% in Bejar et al.¹⁹ vs. 82% in Heuser et al.²³). This difference could, in part, be explained

by tissue source and variant calling methodology, among others. First, our study sequenced 26 genes compared to 40 genes in Bejar, et al.¹⁹ Second, despite improvement in deamination mutations secondary to FFPE by UDG treatment, more stringent variant calling criteria were required to confidently differentiate true mutations from FFPE artifact. These include a VAF threshold of 10% and the addition of *in silico* functional variant analysis. There are other clinical reasons to explain the low incidence of mutated *TP53* in our study *vis-à-vis* Behar et al.¹⁹ For example, in our study only 27% had poor (25%) or very poor (2%) cytogenetic risk according to R-IPSS. In contrast, in Bejar et al. 49% of patients had either -7/7q or +1 ($\pm$ other) (17%) or a complex karyotype (32%).¹⁹ Svobodova et al. showed a strong association between mutated *TP53* and complex karyotype.²⁴ Similarly, Kulasekararaj et al. reported *TP53* mutations in 72% of MDS with complex karyotype and existing 5q abnormalities.²⁵ In our population, 82% had been previously treated with azacitidine which leads us to speculate whether hypomethylating agents might, perhaps, affect the incidence of such mutations.

In our analysis we also identified *IDH2* mutation as a predictor of poor post-transplant outcomes. Patients harboring mutated *IDH2* had a median survival of only 8.4 months and a 3-year PFS and OS of 0. Heuser et al. using Illumina high-throughput sequencing in 308 patients (MDS=47%, secondary AML from MDS=53%) who received and all-HCT reported an incidence of mutated *IDH2* of 4.2%.²³ Mutated *IDH2* was a prognostic predictor for high-risk of relapse and inferior post-transplant survival.²³

While mutations in *ASXL1* occurred within the highest incidence (n=7,8%) in our study sample, its presence did not affect adversely post-transplant OS (HR=0.90 (95%CI=0.32, 2.51), P=0.84). Intriguingly, previous studies have shown inferior OS for mutated *ASXL1* in the non-transplant setting.^{1, 26} It is plausible that allo-HCT overcomes the adverse effect of mutated *ASXL1*; however, large randomized controlled studies comparing an allo-HCT (or not) in mutated *ASXL1* MDS would be necessary to answer this question.

We did not identify a correlation between presence of mutations (>0) and older age (empirically defined as ≥ 60 years). Presence of the following mutations did not affect OS in univariate analysis: *DNMT3A* (p=0.74), *KRAS* (p=0.23), *RUNX1* (p=1.0), *TET2* (p=0.94), *SRSF2* (p=0.62), *SF3B1* (p=0.47), *EZH2* (p=0.46), *CBL* (0.12), *MLL* (p=0.19), *ZRSF2* (p=1.0), *U2AF1* (p=0.37), *KIT* (p=0.44), or *WT1* (p=1.0).

Novel therapies targeting specific mutations are becoming available. Stein et al. demonstrated the safety and efficacy of AG-221, a potent inhibitor of mutant *IDH2*, in 128 evaluable cases of relapsed or refractory AML.²⁷ Fifty two (41%) achieved an objective response (CR=18%).²⁷ It is very likely that therapies such as AG-221 or other genetically-driven treatments will be studied in the setting of early post-transplant consolidation and maintenance provided that they have acceptable toxicity. Moreover, novel approaches are required to effectively target mutated *TP53*.

Rapidly expanding knowledge pertaining to the prognostic significance of various gene mutations has only begun to augment disease-risk prognostication for MDS. This study furthers the implementation of clinical genomics in this disease and will help incorporate

novel therapies, for example *IDH2* inhibitors or others, in the post-transplant setting aimed at reducing the risk associated with these mutations.

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Figure 1.
Overall survival (3-year) in mutated *TP53* compared to wild type *TP53* MDS

Figure 2.
Overall survival (3-year) in mutated *IDH2* compared to wild type *IDH2* MDS

Figure 3.
Overall survival (3-year) in MDS with >0 mutations vs. none

Table 1

Patient, disease, and treatment characteristics

Variables	N= 89
Recipient median age (range), yrs	58 (22–74)
Recipient gender	
Male	52%
WHO at diagnosis	
RA	2%
RARS	3%
RCMD	18%
RAEB-1	35%
RAEB-2	29%
MDS-unclassified	5%
MDS/MPN	1%
MDS-RS	6%
Azacitidine before allo – HCT	
Yes	82%
No	18%
Best response to azacitidine pre – HCT	
CR	9%
CR marrow	4%
PR	13%
HI	6%
SD	44%
PD	6%
Missing	19%
Donor source	
MRD	34%
MUD	48%
MMUD	15%
Cord blood	3%
Cell source	
PBSC	96%
BM	1%
Cord blood	3%
Preparative regimens	
FLU-BU	91%
FLU-MEL	6%

Variables	N= 89
FLU-CY-TBI (2Gy)-ATG*	3%
Intensity of the conditioning regimen	
MAC	91%
RIC/NMA	9%
GVHD prophylaxis	
TAC-MTX	64%
TAC-SIR	21%
TAC-MMF	15%
R-IPSS at time of allo-HCT	
Very low	17%
Low	18%
Intermediate	18%
High	22%
Very high	21%
Missing	3%
Cytogenetic scoring	
Very good	0
Good	56%
Intermediate	15%
Poor	25%
Very poor	2%
Missing	3%
Mutation present >0	
Yes	44%
No	56%

Abbreviations: M: male; WHO with excess blasts-1; RAEB-2: refractory anemia with excess blasts-2; MPN: myeloproliferative neoplasms; CR: complete remission; PR: partial response; HI: transfusion independence; SD: stable disease; PD: progressive disease; MRD: matched-related donor; MUD: matched-unrelated donor; MMUD: mismatched-unrelated donor; PBSC: G-CSF mobilized peripheral blood stem cells; BM: unmanipulated bone marrow; FLU: fludarabine; BU: busulfan; MEL: melphalan; CY: cyclophosphamide; TBI: total body irradiation; MAC: myeloablative conditioning; RIC: reduced intensity conditioning; NMA: non-myeloablative conditioning; TAC: tacrolimus; MTX: methotrexate; SIR: sirolimus; MMF: mycophenolate mofetil

FLU-BU (n=81) cumulative busulfan AUC ranging from 3500–7500 $\mu\text{mol}\cdot\text{min}/\text{L}$ (3500 (n=15), 5300 (n=59), 6000 (n=4), and 7500 (n=3))

*
Regimen used for cord blood allografting.

Table 2

Multivariate analysis (adjusted for percentage BM myeloblasts, presence >0 mutation, and R-IPSS).

variables	3 – year PFS		3 – year OS	
	HR (95%CI)	p – value	HR (95%CI)	p -value
Recipient age (years)		0.63		0.64
< 60	–		–	
60	1.13 (0.70, 1.83)		0.86 (0.46, 1.62)	
BM myeloblasts (%) at time of allo-HCT		0.83		0.60
<5%	–		–	
5%	0.93 (0.49, 1.77)		0.80 (0.35, 1.82)	
R-IPSS score		0.55		0.34
Intermediate	–		–	
High	1.21 (0.65, 2.22)		1.48 (0.67, 3.28)	
Presence >0 mutation		0.33		0.30
No	–		–	
Yes	0.75 (0.41, 1.35)		0.65 (0.29, 1.46)	
<i>TP53</i> mutation		0.06		0.03
No	–		–	
Yes	3.03 (0.96, 9.60)		3.82 (1.12, 13.09)	
<i>IDH2</i> mutation		0.02		0.02
No	–		–	
Yes	4.34 (1.33, 14.13)		4.74 (1.33, 16.91)	

Abbreviations: HR: hazard ratio; PFS: progression-free survival; OS: overall survival; BM: bone marrow; R-IPSS: revised international prognostic scoring system

Bold denotes statistical significance