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Safety, outcomes and T cell characteristics in patients with relapsed or refractory MDS or CMML treated with atezolizumab in combination with guadecitabine

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Abstract

Purpose: We hypothesized that resistance to hypomethylating agents (HMAs) among patients with myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia (CMML) would be overcome by combining a PD-L1 antibody with an HMA.

Materials and Methods: We conducted a phase 1/2, multicenter clinical trial for patients with MDS not achieving an IWG-response after at least 4 cycles of an HMA ("refractory") or progressing after a response ("relapsed") with 3+ or higher risk MDS by the revised International Prognostic Scoring System (IPSS-R) and CMML-1 or -2. Phase I consisted of a 3+3 dose escalation design beginning with guadecitabine at 30 mg/m^2 and escalating to 60 mg/m^2 days 1–5 with fixed-dose atezolizumab: 840mg IV days 8 and 22 of a 28-day cycle. Primary endpoints were safety and tolerability; secondary endpoints were overall response rate (ORR) and survival.

Results: Thirty-three patients, median age 73 (range 54–85), were treated. Thirty patients had MDS and 3 had CMML, with 30% relapsed and 70% refractory. No DLTs were observed in Phase I. There were 3 (9%) deaths in ≤30 days. Five patients (16%) came off study for drug-related toxicity. Immune-related adverse events (IRAEs) occurred in 12 (36%) patients (4 grade 3, 3 grade 2, 5 grade1). ORR was 33% (95% CI: 19, 52%) with 2 complete remission (CR), 3 hematologic improvement (HI), 5 marrow CR, 1 partial remission (PR). Median overall survival (mOS) was 15.1 (95% CI: 8.5, 25.3) months.

Conclusion: Guadecitabine with atezolizumab has modest efficacy with manageable IRAEs and typical cytopenia-related safety concerns for patients with R/R MDS and CMML.

INTRODUCTION

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell malignancies whose incidence increases with age.[1] Among the few treatment options available for higher-risk MDS (HR-MDS), hypomethylating agents (HMAs) such as azacitidine and decitabine are relatively safe and effective in approximately half of patients; however, responses are not durable and median overall survival after failure of an HMA is 4–6 months.[2–4]

The mechanisms of resistance to HMAs are not entirely clear. Tumor cell intrinsic factors such as changes in azanucleoside uptake and metabolism and cell cycle quiescence have been suggested, and several studies in patients indicate that changes in the microenvironment may also play important roles.[5,6] Our group observed that azacitidine-

induced demethylation and upregulation of programmed cell death protein 1 (PD-1) in peripheral T cells was associated with a dismal outcome in patients with HR-MDS.[7] Others have identified upregulation of programmed death-ligand 1 (PD-L1) in CD34+ myeloblasts of patients with MDS after treatment with HMAs.[8] In addition, it has been shown that T cell receptor (TCR) clonotypes change after treatment with HMA.[9] Taken together, these findings suggest that HMAs may engender resistance through unintended dampening of the T cell response to the malignant cells in MDS.

We hypothesized that immune checkpoint inhibition could overcome HMA resistance in patients with MDS or chronic myelomonocytic leukemia (CMML) who were refractory to HMA therapy or had relapsed after responding. To investigate this, we conducted a phase I/II study combining a novel decitabine prodrug, guadecitabine, with a PD-L1 inhibitor, atezolizumab. We chose guadecitabine based on early phase studies in which this drug demonstrated a longer duration of in vivo exposure to the active drug and promising clinical results. Subsequent large phase III clinical trials failed to demonstrate superiority to other HMAs in the treatment of MDS and acute myeloid leukemia (AML) so the drug is no longer being further developed.

MATERIALS AND METHODS

Trial design

This study was a multi-center open-label Phase I dose-finding sub-study followed by a Phase II efficacy study (clinicaltrials.gov identifier NCT02935361) evaluating the combination of guadecitabine with atezolizumab for the treatment of patients with HR-MDS or CMML relapsing after or refractory to at least 4 cycles of at least one HMA. HR-MDS was defined as intermediate, high or very high risk by the revised-International Prognostic Scoring System (IPSS-R)[10] and CMML was categorized as CMML-1 or CMML-2 by the WHO 2016 criteria applied at the time of study entry.[11] While the IPPS-R is not validated among patients at relapse, we considered it an appropriate strategy to exclude lower risk patients at time of study entry. The protocol was approved by the Institutional Review Board at each participating institution and written informed consent was obtained from each patient; the study was conducted in accordance with Good Clinical Practice and the Belmont Report. The study drugs, guadecitabine and atezolizumab, were provided by Astex Pharmaceuticals, Inc. (Pleasanton, CA, USA) and Genentech Inc. (South San Francisco, CA, USA), respectively. Astex ceased manufacturing of guadecitabine and all available product expired 10 months after the last patient was enrolled on this study. The 3 patients who were on study after that received decitabine as per standard FDA prescribing information in lieu of guadecitabine.

Phase I followed a modified 3+3 dose escalation design beginning with 50% of the recommended dose level (DL) of guadecitabine, 30 mg/m^2 (DL 0), given subcutaneous daily for 5 consecutive days, in combination with a fixed dose of atezolizumab, 840 mg given IV on Days 8 and 22 of a 28-day cycle. Guadecitabine would be escalated to 60 mg/m^2 (DL 1) if no dose-limiting toxicities (DLTs) occurred in the first 3 patients on DL 0, expansion of the cohort to 6 would occur for 1 DLT and cessation of further treatment would occur if 2 or

3 patients experienced DLTs. If a DLT occurred in 2 or more patients in the DL 1 group, the dose would be de-escalated to 45mg/m^2 (DL-1) and the same rules applied as for DL 1.

In Phase II, patients received 60mg/m^2 guadecitabine subcutaneously daily for 5 days of a 28-day treatment cycle along with a fixed dose of 840mg atezolizumab IV on Days 8 and 22. Guadecitabine could be held or dose-reduced to manage toxicity. Atezolizumab toxicity was managed based on the FDA prescribing information and investigator's brochure. Efficacy was measured by overall response rate (ORR) based on the 2006 International Working Group (IWG) Response Criteria for MDS.[12] Overall survival (OS) and progression free survival (PFS) were secondary endpoints.

Patient eligibility

Inclusion criteria were: i) age ≥18 years and able to consent; ii) diagnosis of MDS according to the 2016 WHO criteria, of Intermediate or higher risk (3+) by IPSS-R criteria, or CMML-1 or CMML-2; iii) prior treatment with at least 4 cycles of azacitidine and/or decitabine with either disease progression by 2006 IWG criteria[10] ("refractory") or loss of a previously documented response ("relapsed") and ineligible for allogeneic stem cell transplant; iv) ECOG performance status of 0–2; v) adequate hepato-renal function (creatinine <2.5 × upper limit of normal; bilirubin <2.5 × upper limit of normal, transaminases < 3 × upper limit of normal); vi) willingness to use contraception or abstain from childbearing.

Exclusion criteria were: i) active autoimmune disease other than vitiligo, type 1 diabetes, or controlled hypothyroidism; ii) interstitial lung disease, or any disease requiring supplemental oxygen; iii) history of pneumonitis or fibrosis from any cause; iv) active treatment for other malignancies except hormonal therapy for stable breast or prostate cancer; v) history of allergy to compounds of similar chemical or biologic composition as HMAs or to humanized antibodies or fusion proteins vi) >30% blasts in marrow or total white blood cell count >25 × 10^3/L; vii) active infection within 72 hours, need for antibiotics within 7 days or life-threatening infection within 28 days; treatment with investigational agent within 28 days; viii) ongoing treatment with >10mg prednisone or other immunosuppressive or immunostimulatory agent; ix) prior allogeneic stem cell or solid organ transplantation, regardless of duration since transplant; x) prior treatment with any immune checkpoint inhibitor.

Statistical analysis of clinical data

Standard descriptive statistics were used to summarize baseline and study results including toxicity data. The observed overall response rate for the first 6 cycles and during all time on treatment was calculated as the ratio of the number of patients who experienced complete remission (CR), marrow CR (mCR), partial remission (PR) or hematological improvement (HI) divided by the total number of patients treated on study, and its associated binomial 95% confidence interval was calculated.[13] The association of baseline characteristics with response to study treatment was tested by using Fisher's exact test. OS was calculated from the date of treatment start to death due to any cause or to the latest follow-up. PFS was calculated as the time from treatment start to the date of progression or death whichever

observed first, or to the latest follow-up. Kaplan-Meier plots were used to estimate the probabilities of OS and PFS. The associated 95% confidence intervals were calculated using Greenwood's standard errors formula. Log-rank test was used to test the association of baseline clinical characteristics with OS and PFS. Cox proportional model was used for multivariable analysis including all variables with $p \leq 0.20$ in the univariate analysis in the model. Hazards ratios were used to calculate the relative risks of death and likelihood ratio test was used to test the associate of baseline characteristic with OS and PFS in the multivariable analysis. Landmark method was used to test the association of response and OS. For analysis of the impact of correlative data on outcome, including mutations and T cell characteristics, we employed an additional category of "long-survivors" or "short-survivors" whose survival from C1D1 was greater than/equal to, or less than, 15 months respectively. All reported p values were two-sided and p values ≤0.05 were considered statistically significant. All analyses were conducted using SAS statistical software (version 9.4; SAS Institute Inc, Cary, NC).

Isolation of bone marrow mononuclear cells, DNA extraction, targeted sequencing and flow cytometry

Bone marrow mononuclear cells (BMMCs) were isolated at screening and within 96 hours prior to Day 1 of Cycles 3, 5 and 7, and $CD3^+$ cells (T cells), CD34+ cells, and the CD3- and CD34-depleted population was isolated using magnetic beads (for details see Supplemental Methods).

Targeted sequencing was performed on DNA from BMMCs (depleted of CD3+ and CD34+ cells) and BM CD3+ T cell from patients who had sufficient extracted DNA and had received at least 1 cycle of treatment. A custom Twist panel consisting of 145 genes related to myeloid cancer was applied, and samples were sequenced on an Illumina NextSeq 500 instrument at an average target coverage of 600x-1000x per sample (for details see Supplemental Methods). We focused on the 40 genes most commonly mutated in MDS for analysis (Supplementary Table 1). The lower level of detection in the BMMCs was set to a variant allele frequency (VAF) of 2% for all genes, except TP53, for which we used a cut-off of 1%. In the separated CD3+ T cell population we confirmed 90% or greater purity in selected cases. To avoid any false positivity due to contamination by myeloid cells after cell sorting, we only report mutations with a VAF≥ 10% in the sorted T-cells.

Peripheral blood mononuclear cell (PBMC) samples were analyzed using multicolor flow cytometry to evaluate immune activation and correlation of treatment outcome with established T cell and NK cell phenotype and activation panels.[14] Phenotype panels of T and NK cells consisted of fluorophore-labelled antibodies recognizing cell surface markers of CD4+, CD8+, and NK cells (Panel 1 and Panel 2), and the T cell activation panel included fluorophore-labelled antibodies specific to cell surface and intracellular T cell activation markers (Panel 3). Based on sample availability, we evaluated up to three different time points from each of 21 patients: pre-treatment (defined as "PRE"), during the second treatment cycle (C2D1 or C2D8; defined as "EARLY"), and after the third or fourth treatment cycle (C4D1, C4D8 or C5D1; defined as "LATE").

Data Availability Statement

DNA sequencing data and the remainder of the dataset may be requested, pursuant to local IRB guidelines, by emailing the corresponding author.

RESULTS

Patients

Between November 2016 and October 2020, 33 patients (22 men and 11 women) from 4 centers in the United States were enrolled in the study. Demographics and disease characteristics are shown in Table 1. The median age at enrollment was 73 (range, 54–85) years. At study entry, thirty patients had MDS, primarily higher-risk (17 High, 2 Very High, 11 intermediate) by the r-IPSS; a majority (70%) had HMA-refractory disease, as opposed to HMA-responsive disease with relapse (30%), prior to enrollment. Two patients had CMML-1 and 1 patient had CMML-2. Prior regimens included azacitidine monotherapy (31 patients), decitabine monotherapy (8 patients), guadecitabine monotherapy (1 patient), lenalidomide monotherapy (6 patients), low-dose cytarabine monotherapy (2 patients), and induction chemotherapy (1 patient). Six (18.1%) patients received sequential HMAs prior to study entry. Bone marrow blast count at study entry was available for 32 patients: it was >5% in 16 patients (50%), 2.1 to 5% in 10 patients (31%), and ≤2% in 6 patients (19%).

Treatment and Outcomes

Patients received a median of 6 treatment cycles (range 1–22). ORR by 6 months was 30% (95% CI 16%, 49%) with 1 CR (3%); 3 (9%) patients achieved hematologic improvement (2 neutrophil, 1 erythroid), 5 (15%) achieved marrow CR and 1 achieved partial remission (Table 2). One additional patient achieved CR after 6 months for a best on-study ORR of 33% (95% CI: 19%, 52%) with CR rate of 6%. Median duration of response among the 11 responders was 10.1 (range 1.8 – 47.6) months. A statistically significant association was found between female gender and response, p=0.018. There was no significant association of any other baseline characteristics with response to the study treatment.

With a median follow-up of 30.1 (range 11.1 – 51.6) months for the entire cohort (Table 2), and one patient still on treatment as of this publication, median overall survival (mOS) was 15.1 months (95% CI: 8.5, 25.3). Median PFS was 7.2 months (95% CI: 3.6, 13.8). The impact of baseline characteristics on survival is shown in Table 3. Patients under 75 years of age lived significantly longer (mOS 20.3 months, 95% CI: 8.5, 51.6+) than older patients (mOS 9.5 months, 95% CI: 1.5, 15.1, p =0.030). There was a trend toward better survival among women (mOS 25.3 months 95% CI: 9.5, 51.6+) compared to men (8.5 months 95% CI: 3.3, 20.3). Among the 30 patients with MDS, mOS was 16.4 (95% CI: 9.5, 27.3) months, superior to that of patients with CMML (2.1 months 95% CI 0.9, 15.1). Median OS for patients who achieved an IWG response was 19.8 months (95% CI 7.6, 49.7+), which was not statistically superior to that of non-responders whose mOS was 8.7 months (95% CI 6.4, 25.4, p=0.12); individual outcomes are shown in Figure 1. After adjusting by other variables in the Cox proportional hazards model, age (HR for ≥75 3.34, 95% CI: 1.25, 8.97, p=0.022), gender (HR for males 3.30, 95% CI: 1.20, 9.02, p=0.018) and number of prior chemotherapy regimens (HR for >1 regimen 3.29, 95% CI 1.24, 8.74,

p=0.019) were significantly associated with survival. Gender and number of prior regimens were also significantly associated with progression free survival after adjusting for other variables in the Cox proportional model. There was no statistically significant association found between overall or progression-free survival and baseline blast count, IPSS-R score, relapsed vs refractory disease or ECOG performance status at baseline.

Safety

There were no dose-limiting toxicities among the three patients treated at DL −1 in phase 1 nor in the first three treated at DL 1. Among the entire patient population there were three deaths (9%) at ≤30 days of first administration of study drug.One patient with high-risk MDS and grade 4 neutropenia at baseline received days 1–8 and subsequently developed sepsis; he opted to withdraw all therapy and died at day 23. A second patient with CMML-1 at baseline presented for day 1 of treatment with extreme leukocytosis, received days 1–8 of study therapy along with hydroxyurea, and was subsequently found to have brain lesions concerning for leukemic infiltrates. He opted to withdraw all therapy on day 10 and died at home on day 30. The third patient had high risk MDS and grade 4 thrombocytopenia at baseline; he received days 1–8 of study therapy and present with headache and neutropenic fever on day 16 for which he was treated with broad-spectrum antibiotics. He had two episodes of convulsions prompting treatment for presumed encephalitis with steroids and died on day 21 due to brainstem herniation after a lumbar puncture. Cerebrospinal fluid analysis and imaging were felt to be inconsistent with autoimmune encephalitis. However, the neutropenic sepsis was considered likely due to study treatment.

Grade 4 toxicities of any attribution occurred in 21 (64%) patients. The most common study drug-related toxicities were thrombocytopenia (39%), neutropenia (30%), and anemia (30%) (Supplementary Table 2). Five patients (16%) came off study for drug-related toxicity. Immune-related adverse events (IRAEs) occurred in 12 (36%) patients: 2 with grade 3 autoimmune encephalitis, 2 with grade 3 pneumonitis, 1 with grade 2 arthritis, 1 with grade 2 hypothyroidism, 1 with grade 2 colitis, 4 with grade 1 rash, and 1 with grade 1 hyperthyroidism (Supplementary Table 3). One of the 2 patients with presumed grade 3 autoimmune encephalitis was treated with steroids; both patients recovered and were taken off the study. One was a 62 year-old male with very high risk MDS who died at 4 months, and the other was a 71 year-old female with high risk MDS who died at 9.5 months. Of the two patients with autoimmune pneumonitis, 1 was an 81 year-old male with intermediate risk MDS and baseline grade 4 thrombocytopenia who was treated with steroids but died within 1 week in multiorgan failure due transfusion-associated circulatory overload; the other was a 68 year-old male with high-risk MDS who recovered without therapy and was alive at 24.6 months. They developed the complication during cycle 2 and cycle 1, respectively. The median time to develop an IRAE was 1.2 (range 0.2–9.4) months, after a median of 4 (range 1–15) doses of atezolizumab with a median time to recovery of 17 (range 1–28) days (Supplementary Table 3).

Mutational Analysis

DNA was available for genetic analyses in 27 of the 33 patients enrolled in the trial.

BMMCs: All patients except one carried somatic mutations in BMMCs (Figure 2). The median number of mutated genes was 3 (range 0–9), and some genes carried more than one mutation. The most frequently mutated genes were the epigenetic regulators *ASXL1* (12 patients) and *EZH2* (7 patients), with less frequent mutations in *TET2* (6), *DNMT3A* (5) and *IDH1*(2). Mutations in *RUNX1* (8) were also common. Among classical tumor suppressors, *TP53* (4) and *PTPN11* (3) were the most frequently mutated. No characteristic pattern was seen in the distribution among short and long survivors.

CD3+ T cells: Mutations in ≥20 % of the T cell population were frequently observed, particularly in epigenetic regulators and splicing factor-encoding genes (Figure 2). Among 27 patients with mutation data available, mutations were present in 9 of 15 "long survivors" and in 6 of 12 "short survivors;" there was no significant difference between the groups (p=0.7, Fisher's test).

ASXL1 was the most frequent mutation detected; in 7 patients it was found only in the myeloid cells and in 5 it was identified in both the myeloid and the T cell populations ("shared"). While there was no significant association between the mutation and response to the study treatment, the median survival of the patients with shared mutations was not reached whereas it was 16.4 months (95% CI 3.3, 27.3) among those without the mutation (n=15) and 9.5 months with the myeloid-only mutation (95% CI 2.1, 35.0) (Supplementary Figure 1). This was statistically significant after adjusting by age at study entry, gender, diagnosis and the number of prior chemotherapy regimens using the Cox proportional hazards model (p=0.001, based on likelihood ratio test). There were insufficient occurrences of the other mutations in the different cell populations to determine their effect on survival.

Immune profiling

We evaluated lymphocytes with a focus on subset frequencies and activation and proliferative characteristics of both T cells and NK cells (Supplementary Figure 2 and Figure 3). We identified the T effector memory subset of CD8+ T cells (T_{EFF}: CD45RA+, CD197−) as the most frequent subset of CD8+ T cells at baseline and throughout treatment. We observed no differences in the frequencies of CD4+ or CD8+ memory T cells (CM: CD45RA-CD197+; EM: CD45RA−, CD197−; T_{EFF}:CD45RA+, CD197−) at baseline or throughout treatment in terms of response or survival. The frequency of T_{EFF} cells out of the total pool of CD8+ T cells tended to be higher in patients at all 3 timepoints with longer overall survival (≥15 months), though this was not statistically significant (Figure 3A).

While the T_{EFF} cell subset seemed enriched in long survivors, no treatment-induced change was observed in the subsets expressing T cell activation markers such as CD39, CD69 or PD-1. On the contrary, in the short survivors a higher fraction of the T_{EFF} cell subset expressed PD-1, and there was also a tendency for increased expression of CD69 and CD39 (Figure 3B). The expression of Ki67 (a proliferation marker) was also increased in the T_{EFF} cell subset of the short survivors (Figure 3C). This difference between short and long survivors was even more pronounced for T_{EFF} cells expressing both PD-1 and Ki67 (Figure 3C). PD-1 surface expression on CD8+ T cells was upregulated in the short survivor group (Figure 3D). We did not find any correlation between $CD8^+$ T cell activation and T cell mutations identified in the two groups. Furthermore, no differences were observed at the

baseline or post-treatment in the frequency and proliferation of CD4 memory and regulatory (Tregs) subsets.

Given the impact of gender on survival we also analyzed the functional activation of CD8+ T cells for males and females by comparing baseline and post-treatment expression of PD-1 (frequency and intensity), CD39 and CD69 (Supplementary Figure 3). We noted higher expression and intensity of PD-1 and lower expression of CD39 and CD69 at baseline among females with no change over the course of treatment, whereas males had lower baseline expression and intensity of PD-1 and a significant upregulation after treatment. CD39 and CD69 expression was higher in males at baseline and throughout treatment and did not appear to change significantly with therapy.

We found no differences in NK cell ($CD56^{dim}$ $CD16^{+}$) frequencies or treatment-induced change in activation markers, although patients with short survival tended to display more activated NK cells (Supplementary Figure 4).

DISCUSSION

In this study, we combined guadecitabine, a novel HMA, with an immune checkpoint inhibitor (ICI) of PD-L1, atezolizumab, based on translational data suggesting that upregulation of PD-1 in T lymphocytes by azacitidine contributes to HMA resistance and is associated with poor outcome.[5] Our patient population consisted of older patients with HMA-relapsed and refractory MDS and is representative of the epidemiology of MDS in the US (Supplementary Table 4). The tolerability of the regimen was comparable to that seen in solid tumor trials of immunotherapy combinations with 36% of patients experiencing immune-related adverse events which were all grade 3 or lower. Early deaths occurred in 3 patients (9%) after days 1–8 of study treatment, two of whom had high-risk MDS and 1 appeared to be transitioning from CMML to AML during the first cycle. Two of these patients developed sepsis and opted to forego further antibiotics and transition to hospice. The third patient developed sepsis with multiorgan failure and died of brainstem herniation after a lumbar puncture. Response rates were modest, with 33% of patients experiencing an IWG-defined response at any time, including 2 with CR. Median survival was 15.1 months in the entire study population and 16.4 months in the 30 patients with MDS. In a multivariate analysis we observed a statistically significant longer overall survival in patients younger than 75, in women, and in patients with only 1 prior treatment regimen (Table 3).

Outcomes after failure of upfront HMA are dismal,[15] and phase II studies of treatments for patients with R/R MDS have not produced mOS beyond 1 year. These include guadecitabine monotherapy and other HMA-ICI combinations. In a phase II study of patients treated with guadecitabine alone, mOS was 11.7 months in the 53 patients with relapsed or refractory MDS or CMML.[16] Another Phase II study administered pembrolizumab, a PD-1 inhibitor, with azacitidine to a similar study population, achieving an ORR of 25% and mOS of only 5.8 months.[17] The addition of venetoclax, a bcl-2 inhibitor, to azacytidine produced a mOS of 11.4 months in a similar patient population.[18]

The tolerability of azacitidine with atezolizumab for treatment of MDS was recently questioned in a clinical trial in which patients experienced significant toxicity, leading to discontinuation of the trial.[19] Early deaths were noted the HMA-naïve cohort whereas in the small cohort of 14 patients with relapsed or refractory MDS, the results were comparable to ours with respect to response rates (28.6%) and deaths (18%). It is unclear if the lower toxicity in the previously HMA-exposed patients is due to lower tumor burden or perhaps a reduction in the number or reactivity of T cell clones leading to fewer autoimmune adverse events.

We did not find immune-related adverse events to be a major cause of death. The most serious were 2 events of autoimmune encephalitis and 2 of autoimmune pneumonitis, all grade 3. In a recent publication, autoimmune pneumonitis occurred in 12% of patients with AML treated on different studies with ICI-containing regimens (nivolumab, a PD-1 inhibitor, and/or ipilimumab, a CTLA4 inhibitor).[20] Almost half of these events were fatal. Male sex and thrombocytopenia were considered risk factors and the development of pneumonitis was associated with a higher risk for mortality overall. While only 2 (6.1%) patients developed autoimmune pneumonitis in our study, both were males and 1 died within a week of starting steroids. Autoimmune encephalitis is more difficult to diagnose accurately than pneumonitis and both of the patients in our study who were treated as such presented with altered mentation and were concurrently treated empirically for infection. In another study combining atezolizumab with an HMA, only 1 case of autoimmune pneumonitis in 46 treated patients was noted, and no cases of autoimmune encephalitis were reported, but there were several reports of autoimmune hepatitis.[19] Given the small sizes and discordant toxicity results from our study and that of Gerds et al, it is difficult to assert that the profile of autoimmune adverse events is unique to the ICI target in patients with myeloid malignancies.

In 27 of 33 patients enrolled in our study, we sequenced a panel of 40 genes commonly mutated in MDS and identified mutations in the myeloid compartment BMMCs (CD3-depleted) and in sorted CD3+ T lymphocytes. To the best of our knowledge the detailed mutational spectrum of T-cells in MDS after treatment with HMAs and/or treated with immune checkpoint inhibitors has not previously been reported, although clonal involvement of the T-cell compartment is recognized.[21] In the myeloid compartment mutations were frequently observed in *ASXL1*, *RUNX1* and *EZH2*, while 4 of 27 had a *TP53* mutation; there was no significant difference in the distributions of myeloid mutations among patients with long and short survival. For T-cell mutation detection we employed a rigorous VAF cut-off of 10% to avoid misleading data due to lack of purity or from very small clones, which may be less likely to affect T cell function. We found that 15 of 27 patients carried mutations in T cells. In all but one case, the mutations detected in T cells were also present in the myeloid compartment, suggesting origination from a common stem cell ancestor. Mutations restricted to the myeloid compartment were more frequent in classical tumor suppressor genes, probably reflecting their origin in later stage progenitors from this compartment. One case showed mutations in the T cell compartment only, which could be a result of more efficient removal of myeloid clones by the previous HMA treatment. We could also have overlooked mutations in smaller T cell subsets; the limited amount of material did not allow for further analyses of specific T cell subsets. Importantly, all patients were previously

treated with HMA in first line, so the high frequency of mutated T cell clones may be a consequence of selection by HMA.

We did not observe any significant difference between long and short survivors with respect to the presence of mutated T cells. However, we did find that *ASXL1* mutations, which have previously been associated with HMA resistance in MDS,[22] were present in both the BMMCs and the T cells of both of the patients who achieved a CR and in 5 of 8 affected long survivors, while *ASXL1* mutations were restricted to the myeloid compartment in all 4 affected short survivors. A multivariate analysis demonstrated superior survival in patients with shared *ASXL1* mutations compared to those with myeloid-only or those lacking *ASXL1* mutations. Notably, *ASXL1* mutations were also associated with improved survival in a study of patients with relapsed and refractory acute myeloid leukemia (AML) treated with azacitidine and the PD-1 inhibitor nivolumab, though their prevalence in T lymphocytes was not investigated.[23] While the impact of each of the commonly occurring myeloid somatic mutations has not been defined in T cells, it is now recognized that both *DNMT3A* and *TET2* disruption can influence the epigenetic profile and promote CAR T-cell function.[24–26] A recent study in mice expressing mutant *ASXL1* in T cells demonstrated increased PD1 expression which may support the efficacy of immune checkpoint inhibition in cancer patients whose T cells carry this mutation. [26] These observations merit confirmation in a larger cohort of patients with *ASXL1* mutations.

We performed a comprehensive flow cytometry assessment of T cells from before and after treatment initiation. As expected, we identified significant upregulation of activation markers in CD8+ T cells, which was more pronounced in men, but we could not correlate this with response to treatment or longer survival. In fact, there seems to be more early activation of CD8+ T cells among short survivors, although the overall fraction of effector CD8+ T cells in this group tended to be lower. We did not have sufficient samples to determine if *ASXL1*-mutated T cells had a unique phenotype at baseline or over the course of therapy. This deserves further investigation. It is also possible that the beneficial impact of PD-L1 inhibition among long survivors is exerted through other cells in the marrow microenvironment, such as myeloid-derived suppressor cells (MDSCs), which are known to express PD-L1.[27]

In conclusion, the combination of guadecitabine and atezolizumab in patients with refractory or relapsed MDS produced a modest response rate and potentially a survival advantage in certain patients with R/R MDS. The toxicity profile was not unexpected with 64% of patients developing grade 4 toxicities and 36% developing immune-related adverse events, all ≤ grade 3. Since the initiation of this study, guadecitabine failed to show superiority to other HMAs in R/R MDS and R/R AML patients in two large phase III trials. This suggests that any benefit achieved by the patients in our study was from the combination, not the guadecitabine itself. Our findings therefore warrant further investigation of the potential for immune checkpoint inhibition to resensitize MDS to any of the available HMAs in R/R MDS and provide an impetus to explore the biologic roles of gender, co-occurring T cell mutations in *ASXL1* and other genes, and myeloid-derived cells in the microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Significance

The addition of an immune checkpoint inhibitor to an HMA for the treatment of R/R MDS and CMML is based on previous work by our group demonstrating azacitidine-induced upregulation of PD-1 on T lymphocytes in patients with MDS receiving first line therapy. Among patients previously treated with and either refractory to or relapsed after HMAs we provide a comprehensive flow cytometric examination of T cell characteristics at study entry and over the course of combination therapy. Moreover, we provide the first description of the prevalence of common myeloid mutations in T cells in patients previously treated with HMA. This is the first trial to demonstrate the potential for enhanced responsiveness to immune checkpoint inhibition among patients harboring the *ASXL1* mutation in their T cells, a finding which will shape patient selection as we move toward optimizing the synergy between epigenetic and immunologic therapies in future trials.

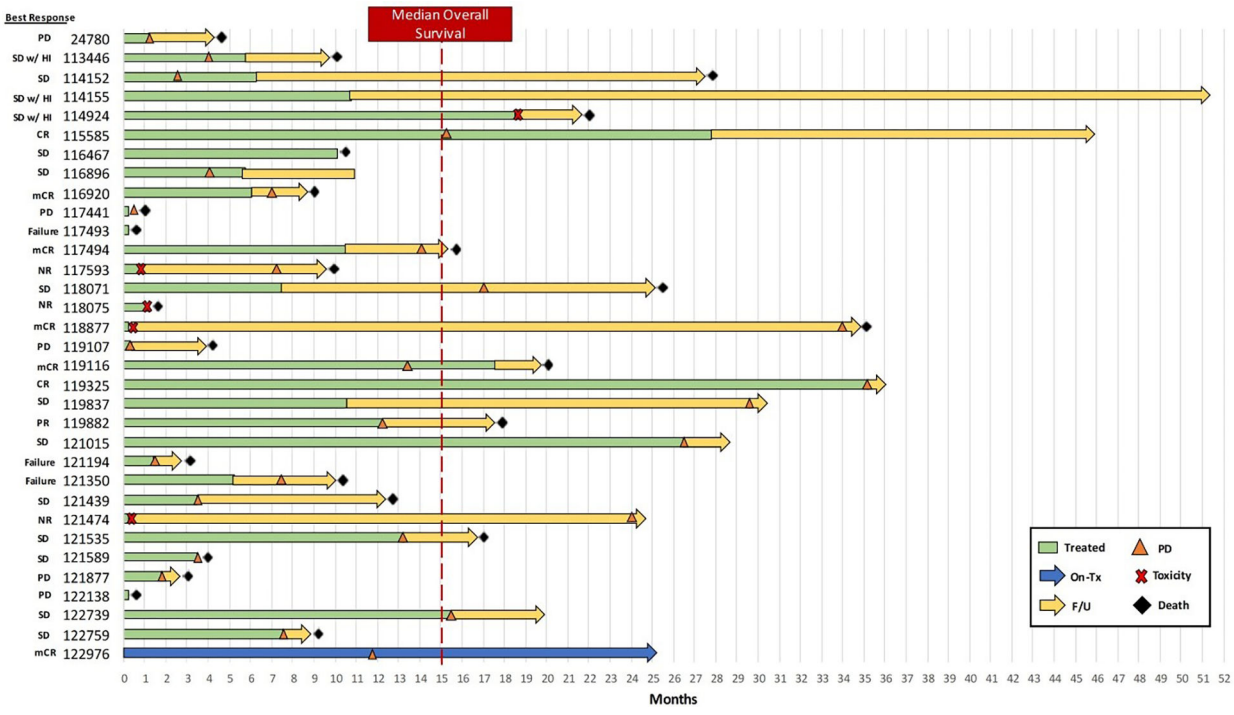

Figure 1.
Swimmer Plot of Patient Outcomes on Guadecitabine and Atezolizumab.

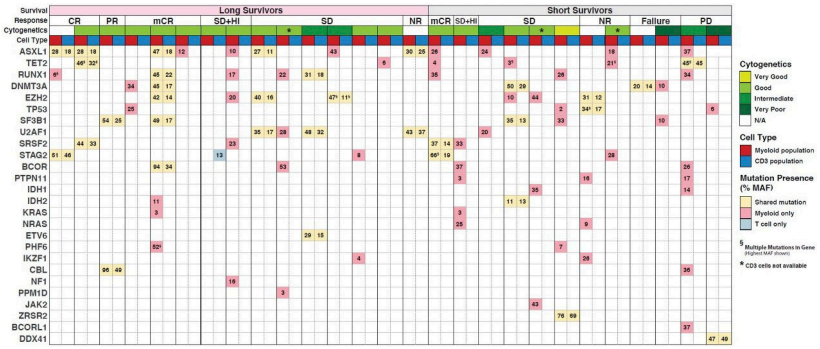

Figure 2.

Mutations in myeloid and T lymphocyte compartments in MDS patients considered short-survivors and long-survivors treated with guadecitabine and atezolizumab

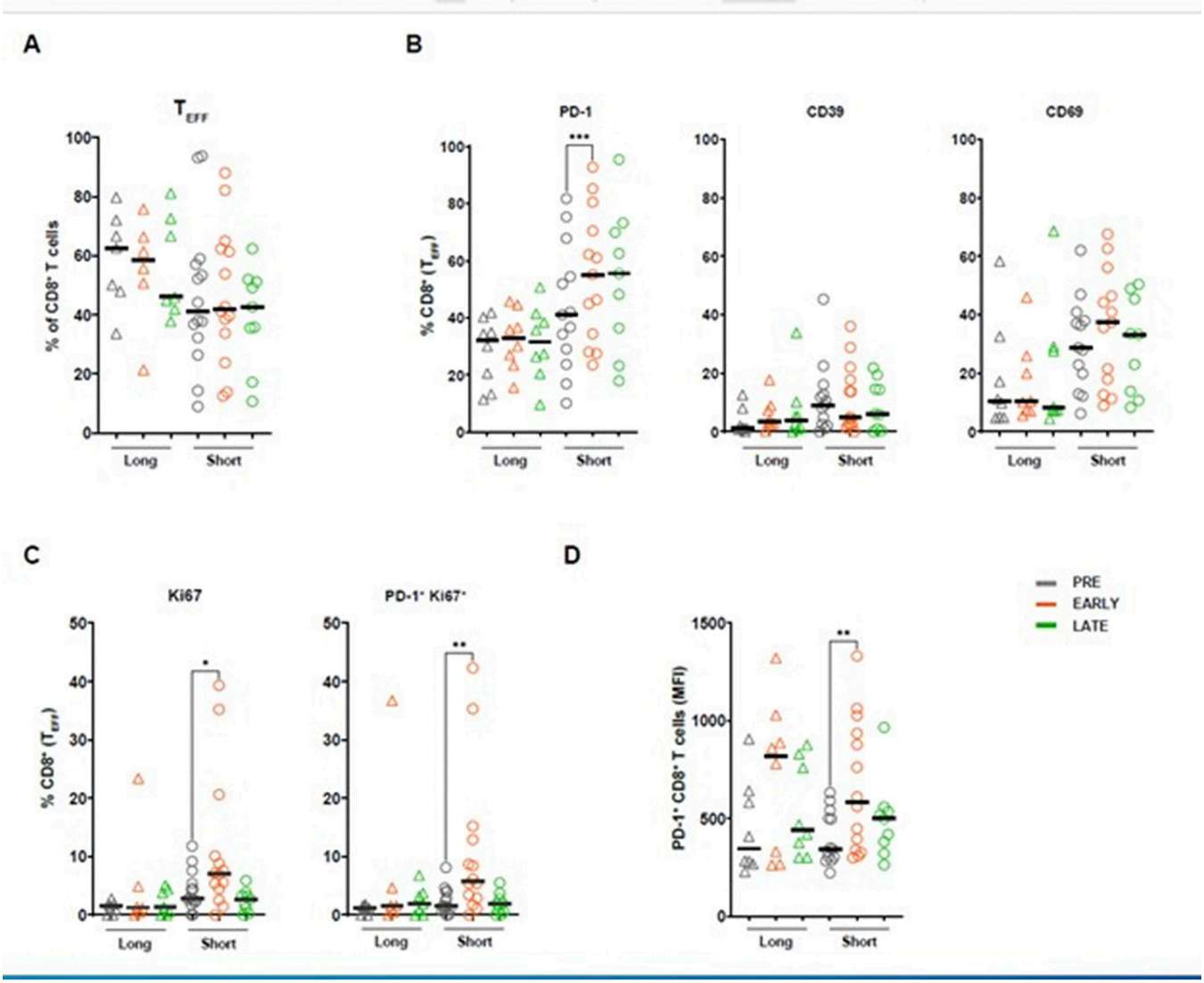

Figure 3. T cell activation in MDS patients considered short-survivors and long-survivors treated with guadecitabine and atezolizumab.

Comparative evaluation of T cell profiles in short- and long-survivors (>15 months) was performed using flow cytometry analysis on PBMCs from 21 patient samples at three different time points; Before treatment (PRE), after second treatment cycle (C2D1 or C2D8; EARLY), and after the third or fourth treatment cycle (C4D1, C4D8 or C5D1; LATE). Gating strategy of flow cytometry analysis is shown in Supplementary figure 1. **A**. $CD8^+$ effector T cell (T_{EFF}) subset in long- and short-survivors. **B**. Expression of cell surface activation markers PD-1, CD39, and CD69 on T_{EFF} $CD8^+$ T cells. Wilcoxon paired t test. Short-survivors, PD-1 p= 0.0002 (PRE vs EARLY) **C**. Proliferation of T_{EFF} $CD8^+$ T cells measured as intracellular secretion of Ki-67 alone or in combination with expression of PD-1 following the treatment in long-and short-survivors. Wilcoxon paired t test. Short-survivors; Ki-67 p= 0.004 (PRE vs EARLY), Ki-67 and PD-1 p=0.004 (PRE vs EARLY) **D**. Change in median fluorescence intensity (MFI) of PD-1 expression on overall $CD8^+$ T cell

population before and after the treatment. Wilcoxon paired t-test. Short-survivors p=0.005 (PRE vs EARLY).

Table 1.

Patient Demographics and Baseline Characteristics

	Number Patients	Percent
Total Patient Enrolled	33*	100
Enrollment Time	11/28/16 – 10/21/20	
Age at Study Entry		
>60	31	94
Median (Range)	73 (54 – 85)	
Gender		
Female	11	33
Male	22	67
ECOG Performance Status at Study Entry		
0	8	24
1	24	73
2	1	3
Diagnosis		
MDS	30	91
CMML-1	2	6
CMML-2	1	3
IPSS-R Prognostic Score for MDS at Study Entry (n=30 MDS)		
Intermediate (Risk Score > 3 and <=4.5)	11	37
High (Risk Score > 4.5 and <=6)	17	57
Very High (Risk Score >6)	2	7
Disease Status at Study Entry		
Refractory Disease	23	70
Relapsed Disease	10	30
Baseline Bone Marrow Blasts (IHC)		
≤ 2.0%	6	19
2.1% – 5.0%	10	31
>5%	16	50
Median (Range)	5.5% (0% – 20%)	
Inadequate Specimen	1	
ASXL1 Mutation		
Shared Mutation	5	15
Myeloid Mutation Only	7	21
No Mutation	21	64
Number of Prior Chemotherapy Regimens		
1	18	55
2	13	39
3	2	6

	Number Patients	Percent
Median (Range)	1 (1 – 3)	
Received Prior Hypomethylating Agent (HMAs)		
Yes	33	100
Both AZA and Dacogen (Sequentially)	6	18
Only AZA	25	76
Only Dacogen	2	6
Median (Range) Cycles of Prior AZA	9 (3 – 61)	
Other Prior Treatment		
Lenalidomide	6	18
Guadecitabine	2	6
Low-dose Cytarabine	2	6
7+3 (ARA-C with Idarubicin)	1	3
Adrimaycin, Cytoxan, Taxotere	1	3
Cisplatin	1	3
Cyclophosphamide	1	3
Doxorubicin	1	3
Vincristine Sulfate	1	3
ASXL1 Mutation Status		
Mutation	12	36
No Mutation	15	45
Not Available	6	18
RUNX1 Mutation Status		
Mutation	8	24
No Mutation	19	58
Not Available	6	18
EZH2 Mutation Status		
Mutation	7	21
No Mutation	20	61
Not Available	6	18
TET2 Mutation Status		
Mutation	6	18
No Mutation	21	64
Not Available	6	18
DNMT3A Mutation Status		
Mutation	12	36
No Mutation	15	45
Not Available	6	18

Table 2.

Treatment and Outcomes

	Number Patients	Percent
Total Cycles of Treatment Received		
1 Cycle	7	21
2 – 5 Cycles	9	27
≥ 6 Cycles	17	52
Median (Range)	6 (1 – 22)	
Total Cycles Treatment Given	238	
Dose Modified		
No	26	79
Yes	7	21
Dose Modified (n=7)		
Atezolizumab	2	
Guadecitabine	5	
Dose Interrupted Due to Toxicity		
No	8	24
Yes	25	76
Evaluable for Response		
Yes	33	100
Best Response During the First 6 Cycles		
CR	1	3
Marrow CR	5	15
PR	1	3
SD with HI	3	9
SD	12	36
PD	5	15
Failure	3	9
Non-Responder	3	9
Response Rate During 1st 6 Cycle (CR, mCR, PR, HI)	10	30 (95% CI: 16%, 49%)
Best Response During Treatment		
CR	2	6
Marrow CR	5	15
PR	1	3
HI	3	9
SD	11	33
PD	5	15
Failure	3	9
Non-Responder	3	9
Overall Response Rate (CR, mCR, PR, HI)	11	33 (95% CI: 19%, 52%)

	Number Patients	Percent
Time Start Response After Treatment Start (n=11)		
Median (Range)	3.9 (1.8 – 15.1) months	
Duration of Response (n=11)		
Median (Range)	10.1 (1.8–47.6) months	
Ever experienced Any Grade 4 Toxicity, Any Attribution		
No	12	36
Yes	21	64
Off Treatment		
No	1	3
Yes	32	97
Off Treatment Reason (n=32)		
Disease Progression/Clinical Deterioration	16	50
Unacceptable Toxicity	5	16
Death While On Treatment	4	13
Physician's Decision	4	13
Patient Withdrawal	3	9
Median (Range) Months on Treatment	5.5 (0.2 – 32.8)	
Median (Range) Months on Study	6.0 (0.2 – 35.2)	
Died within 30 Days after Treatment Start		
Yes	3	9
Overall Survival		
Median (95% CI)	15.1 (8.5, 25.3) Months	
Progression Free Survival		
Median (95% CI)	7.2 (3.6, 13.8) Months	
Follow-Up Time		
Median (Range)	30.1 (11.1 – 51.6) months	

Table 3.

Association of Demographics and Baseline Clinical Characteristics with OS by Univariate and Multivariate Analyses (n=33)

Factors	n	Overall Survival Median (95% CI) (Months)	p-value*	Overall Survival Hazard Ratio (95% CI) Univariate Analysis#	p-value^	Overall Survival Hazard Ratio (95% CI) Multivariable Analysis#	p-value^
Age at Study Entry			0.030		0.043		0.022
<75	23	20.3 (8.5, 51.6+)		1.00		1.00	
≥75	10	9.5 (1.5, 15.1)		2.41 (1.06, 5.48)		3.34 (1.25, 8.97)	
Gender			0.13		0.12		0.018
Female	11	25.3 (9.5, 51.6+)		1.00		1.00	
Male	22	8.5 (3.3, 20.3)		1.94 (0.81, 4.63)		3.30 (1.20, 9.02)	
ECOG Performance Status at Study Entry			0.69		0.69		
0	8	17.3 (0.9, 30.1+)		1.00			
1 or 2	25	12.1 (8.5, 21.6)		1.22 (0.45, 3.29)			
Diagnosis			0.033		0.046		0.45
MDS	30	16.4 (9.5, 27.3)		1.00		1.00	
CMML	3	2.1 (0.9, 15.1)		3.61 (1.02, 12.69)		0.55 (0.11, 2.72)	
IPSS-R Prognostic Score for MDS at Study Entry (n=30)			0.83		0.83		
Intermediate Risk Score > 3 and <=4.5)	11	21.6 (2.5, 46.2+)		1.00			
High or Very High (Risk Score > 4.5)	19@	12.1 (8.2, 35.0)		(1.11 (0.44, 2.76)			
Diagnosis and IPSS-R			0.10		0.21		
MDS – Intermediate Risk	11	21.6 (2.5, 46.2+)		1.00			
MDS - High or Very High Risk	19@	12.1 (8.2, 35.0)		1.11 (0.45, 2.77)			
CMML	3	2.1 (0.9, 15.1)		3.86 (0.96, 15.48)			
Disease Status at Study Entry			0.50		0.51		
Refractory Disease	23	17.3 (8.2, 35.0)		1.00			
Relapsed Disease	10	10.5 (4.0, 21.6)		1.34 (0.57, 3.18)			
Baseline Bone Marrow Blasts (IHC)			0.74		0.73		
≤ 2.0%	6	10.5 (2.5, 46.2+)		1.00			
2.1% - 5.0%	10	16.4 (1.5, 51.6+)		1.31 (0.34, 5.12)			
>5%	16	9.8 (8.5, 27.3)		1.60 (0.45, 5.65)			
Number of Prior Chemotherapy Regimens			0.11		0.12		0.019
1	18	21.6 (8.5, 51.6+)		1.00		1.00	
> 1	15	9.5 (2.1, 17.3)		1.92 (0.85, 4.32)		3.29 (1.24, 8.74)	

[*] p-value based on logrank test

[^] p-value based on likelihood ratio test from Cox proportional hazards model. All variables with p≤0.20 on univariate analysis (age at study entry, gender, diagnosis, and the number of prior chemotherapy regimens) were included in the model for multivariable analysis

[#] Hazard ratio 1.0 is the reference group.