INTRODUCTION

Myelodysplastic syndrome (MDS) is a disease of dysregulated clonal hematopoiesis with defective cell maturation leading to one or more cytopenias. As this type of abnormality increases with age, MDS is the most frequent hematological disease in the elderly. MDS has a very broad cytogenetic and mutational landscape which is being studied in depth in an effort to refine the classification and adapt treatment. Thirty to 40% of MDS cases transform into secondary acute myeloid leukemias (sAML). The transformation to sAML is seen either as the consequence of an accumulation of mutational events or of a single “tipping point” mutation. The most frequently mutated genes can be separated into 6 major pathways of which several are related with chromatin regulation. This are histone modification (ASXL1, EZH2), DNA methylation (TET2, DNMT3A, IDH1, IDH2), signal transduction (RTK, FLT3, KIT, NRAS, KRAS, PTPN11), transcriptional activation (RUNX1, TP53), cohesin complex (SMC1, SMC3, RAD21, STAG1/2), and RNA splicing (SF3B1, SRSF2, U2AF1, ZRSR2).

The only curative treatment for MDS is allogeneic hematopoietic stem cell transplantation, but this intensive procedure comes with immunosuppression and is often unsuitable for elderly patients due to their comorbidities. Thus, alternative treatments are often indicated. The azanucleosides 5-azacitidine (AZA), and 5-aza-2’-deoxycytidine (decitabine) are currently the best treatment options for high-risk patients noneligible for stem cell transplantation (reviewed in Diesch et al11). However, half of the patients do not respond to treatment with azanucleosides and the other half eventually acquires resistance leading to relapse. Numerous studies have explored mechanisms of resistance to azanucleosides or biomarkers to predict response to treatment. For example, Tet methylcytosine dioxygenase 2...
(TET2), which is involved in DNA demethylation, is frequently mutated in MDS and the prognostic value of TET2 mutation has been controversially discussed, as it has been associated with both favorable and worse prognostics. 14,15 Another example is the uridine-cytokine kinase UCK1, which is involved in the cellular metabolism of AZA. Knockdown of UCK1 has been shown to impair AZA response in vitro, and conversely, higher expression was associated with prolonged overall survival in AZA-treated MDS patients. 16

Combining therapies is the most promising strategy to overcome or avoid the problem of acquired resistances. In the case of AZA, the BCL2 inhibitor venetoclax increases its sensitivity and has recently been approved for the treatment of newly diagnosed AML patients not suitable for intensive chemotherapy. 17 Similarly, combination of AZA and the mutant p53 inhibitor, APR-246, showed promising results in MDS patients with TP53 mutations. 18 We have previously reported that the inhibition of the histone acetyltransferase CBP was synergistic with AZA in sAML cells. 19 This was dependent on the RNA-dependent effects of AZA and the combined inhibition of the protein synthesis machinery.

Here, we have taken a dual approach by identifying genes affecting AZA sensitivity in vitro followed by the analysis of their expression and that of other genes in a cohort of samples from MDS patients at diagnosis that subsequently underwent AZA therapy.

METHODS

Plasmids including hEpi9 library

The shRNA library hEpi9 consisting of 7296 shRNA targeting 912 different chromatin genes (8 shRNAs per target) as well as the pPRL-UCEO SFFV-GFP-miRE-PGK-Puro (cSGEP) library, were obtained from the Addgene (Addgene #12260) and pCMV-IVSV-G (Addgene #8454).

Cell culture and drug treatments

MOLM-13 (#ACC 554) and SKK-1 cells have been obtained from DSMZ as a collaboration with Hans Drexlert and been characterized in detail. 20 MOLM-13 has a FLT3 internal tandem duplication (FLT3-ITD), an MLL-AF9 fusion and mutations in the cellular metabolism (FLT3-ITD), an MLL-AF9 fusion and mutations in TET2, JAK2, and harbors mutations in splicing factor SRSF2. We have previously shown that the inhibition of histone acetyltransferase CBP was synergistic with AZA in sAML cells. 19 This was dependent on the RNA-dependent effects of AZA and the combined inhibition of the protein synthesis machinery.

The shRNA screen data generated in this study have been deposited in the GEO database under access code GSE208736.

RESULTS

AZA response modulators include genes encoding components of the ISwi complex

To identify response sensitizers in vitro, we performed a loss-of-function shRNA screen in a sAML cell line. The SKK-1 cell line has been isolated from a patient with MDS-derived sAML and has recently been approved for the treatment of newly diagnosed AML patients not suitable for intensive chemotherapy. 17 Similarly, combination of AZA and the mutant p53 inhibitor, APR-246, showed promising results in MDS patients with TP53 mutations. 18 We have previously reported that the inhibition of the histone acetyltransferase CBP was synergistic with AZA in sAML cells. 19 This was dependent on the RNA-dependent effects of AZA and the combined inhibition of the protein synthesis machinery.

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Flow cytometric analysis of viability

Cells were treated with AZA (Sigma-Aldrich) at the indicated concentration and duration and cell viability was assessed by flow cytometry of cells stained with 1 µg/mL 4',6-Diamidino-2-phenylindole dihydrochloride (Thermo Scientific), and 100 µM MitoTracker Red CMXRos (Thermo Scientific), using the LSR Fortessa cytometer and the BD FACSDiva software (BD Biosciences, Franklin Lakes, NJ). Statistical analysis (ANOVA test) were calculated using GraphPad Prism software (version 6).

Loss-of-function screen

The loss-of-function shRNA screen was performed as previously described. 19 In brief, SKK-1 cells were transduced with the hEpi9 library. To achieve a 1000x representation of the hEpi9 library, considering a 10% infection efficiency, 7.3 × 107 cells were transduced in triplicates. Transduced cells were selected using puromycin and then the mixed population treated with 0.075 µM AZA every 2 days or left untreated for 21 days. Genomic DNA was extracted, prepared and sent for sequencing using the Illumina HiSeq2500 platform (50 bp, single-end, using custom sequencing primers).

Patient sample preparation and nanostaining analysis

Bone marrow samples from patients were obtained from the Munich Leukemia Laboratory (MLL) and originated from the cohort described in Kündgen et al. 11 Samples were stored in 300 µL RLT buffer and β-Mercaptoethanol at ~80°C. RNA extraction was performed using the Qiagen RNeasy kit according to instructions. 100 ng in 5 µL final volume (20 ng/µL) were prepared and 8 µL of mastermix (Reportor barcoded probes + hybridization buffer containing known concentrations of positive and negative controls), and 2 µL of capture probes added and then incubated 20 hours in a thermocycler (65°C, lead at 70°C) for hybridization. The hybridized material was passed to the prep-station where the hybridized molecules are captured on the chip. On the digital analyzer, 553 frames were taken for each sample and each barcoded probe was counted. The analysis was done by the nSolver 4.0 software using default settings and the data normalized to 2 housekeeping genes (GUSB and TUBB).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software (version 6) or R (version 4.1.2) and suitable tests are indicated in figure legends. Survival curves for overall survival were estimated and plotted according to the Kaplan–Meier method and were compared for different genes’ expressions by the log-rank test. The level of significance was set at 0.05. Survival analysis was carried out using Statistical Package for Social Sciences (SPSS) package version 20 for Windows.

Data availability

The shRNA screen data generated in this study have been deposited in the GEO database under accession code GSE208736.
lentivirally infected with the shRNA library at low viral titer favoring single copy integration per cell and aiming for 1000 cells per hairpin. After puromycin selection of infected cells, the polyclonal cells were split into 2 batches, one batch being treated with 0.075 μM AZA every 2 days and the other batch left untreated (Figure 1B). This process was performed in 3 replicates. After 21 days, we extracted genomic DNA and prepared libraries for the high-throughput sequencing of shRNA guide strands. Bioinformatics analyses of the sequencing data were done by comparing the abundance of each shRNA sequence in treated versus untreated samples. We determined hits based on the fold change of abundance, averaging all short hairpins targeting the same gene and following the same trend. We only used those hits for which at least five of the eight shRNAs showed the same trend for further analysis (Suppl. Table S1). Hits fell in 2 distinct categories: genes limiting the survival in the presence of AZA, leading to an enrichment of knockdown cells in treated population, and genes required for the survival in presence of AZA, for which the knockdown was not tolerated and knockdown cells depleted in treated population. Among the top hits of genes inhibiting survival in the presence of AZA, we found the gene ERCC2 encoding for the protein ERCC excision repair 2 (Figure 1C). Conversely, the expression of several genes encoding components of the Imitation SWItch (ISWI) complex including CHRAC1, BAZ1A, BAZ1B, SMARCA5, and POLE3 were required for survival in the presence of AZA and thus their knockdowns were depleted out of the cell population (Figure 1C). To validate the results of the shRNA screen, we generated polyclonal SKK-1 cell lines with stable single knockdown of a subset of hits. To accelerate the validation, we assessed the cell viability after 7 days of treatment with different concentrations of AZA. As shown in Figure 1D, the resistance-conferring effect of ERCC2 knockdown and the sensitizing effect of POLE3 knockdown were visible across all AZA concentrations. Although the knockdown efficiency was variable (Figure 1E), we were able to validate the majority of the other chosen hits (summarized in Figure 1F). Importantly, we confirmed the influence of ERCC2, the ISWI components BAZ1A and CHRAC1 and some

Figure 1. Chromatin regulators affecting AZA sensitivity are identified by a loss-of-function shRNA screen. (A) Titration of AZA in SKK-1 cells to determine concentration used in shRNA screen. (B) shRNA screen workflow. SKK-1 cells were infected with hEpi9 library, puromycin selected, and treated with 0.075 μM AZA every 2 d for 21 d. After gDNA extraction, shRNA guide strands were sequenced and the abundance of shRNAs compared in treated vs untreated samples. (C) Volcano plot of genes corresponding to enriched ("Genes inhibit survival in presence of AZA," blue) or depleted ("Genes required for survival in presence of AZA," red) shRNAs. Top hits selected for Nanostring analysis are named. (D) Representative validation of an enriched and depleted hit. SKK-1 cells stably expressing cSGEP, shERCC2, or shPOLE3 were treated for 7 d with the indicated concentrations of AZA and the percentage of live cells assessed by DAPI/MitoTracker staining. (E) Relative expression of genes targeted with indicated shRNAs compared to the control (cSGEP) by RT-qPCR in SKK-1 cells. (F) Summary of shRNA screen validation. SKK-1 cells stably expressing indicated shRNAs were treated for 7 d with 1 μM AZA or left untreated and the percentage of live cells assessed by DAPI/MitoTracker staining. (G) Relative expression of genes targeted with indicated shRNAs compared to the control (cSGEP) by RT-qPCR in MOLM-13 cells. (H) MOLM-13 cells stably expressing indicated shRNAs were treated for 7 d with 1 μM AZA or left untreated and the percentage of live cells assessed by DAPI/MitoTracker staining. (D, F, H) Data represent mean ± SEM of at least 3 independent experiments. Statistical analysis was performed using 2-way ANOVA. *P value ≤ 0.05. AZA = azacitidine; FC = fold change.
other hits in a second sAML cell line, MOLM-13 (Figure 1G and H). Of note, cells did not tolerate stable depletion of the ISWI ATPase SMARCA5.

Taken together, we identified several genes affecting the sensitivity to AZA in cultured sAML cell lines including genes encoding for ISWI complex components.

Expression of selected genes was assessed in MDS patient cohort

To assess the expression patterns of genes identified in the shRNA screen as well as other selected genes, we obtained a cohort of 36 MDS patient samples, collected between 2004 and 2014 (Figure 2A; Suppl. Tables S2 and S3). These patients were well annotated (Table 1) and part of a larger cohort for which the mutation status of frequently mutated genes had been previously assessed.15 According to WHO 2008,25 20 (56%) had MDS, 2 (6%) had chronic myelomonocytic leukemia type 2, and 14 (39%) had AML/MDS either therapy-related or secondary. In the present cohort, the most frequent mutations were SRSF2 (47%), ASXL1 (42%), and RUNX1 (31%) (Table 1). One patient had no detectable mutations, and 27 patients (77%) had <1 mutation. Regarding cytogenetics, 15 patients (42%) had normal karyotypes, and 1 had only one cytogenetic alteration, all others presented more than one alteration. Among these, 6 of them (17%) had a loss of chromosome 7, 7 (19%) had a 5q deletion, and 4 (11%) a trisomy of chromosome 8. Samples were taken at diagnosis and patients underwent between 3 and 23 AZA treatment cycles with an average of 6.8 cycles. A cycle is defined as 75 mg/m² per day for 7 days every 28 days.

To assess any statistical association between the response to AZA treatment and the expression of genes of interest in patient samples, we set out to study the expression of a panel of genes using Nanostring technology. We designed a Nanostring panel that contained probes recognizing 14 AZA response-modulating genes identified in the described loss-of-function screen (genes indicated in Figure 1C), and the gene PWWP2B that we had identified in a previous screen performed with higher AZA concentrations.19 Based on our previous observation that inhibition of protein acetyltransferases CBP and p300 enhance AZA response in cultured cells,19 we included the encoding genes CREBBP and EP300 and a number of downstream target genes related with protein synthesis in our analysis. Furthermore, the gene panel contained 8 genes known to be involved in AZA metabolism and protein synthesis,5 6 other MDS-related genes, as well as 5 other genes including three housekeeping genes (Figure 2B; Suppl. Table S4).

The Nanostring analysis was performed using RNA extracted from total bone marrow samples collected at diagnosis from the described 36 patients after erythrolytic lysis and without sorting (Figure 2A). The total mRNA count differed by up to 4 magnitudes in-between genes, but much less, around one magnitude, for the same gene between patients (Figure 2B; Suppl. Table S5, normalized to housekeeping genes GUSB and TUBB). Overall gene expression correlation analysis separated the entire set of genes in 2 major clusters including four smaller subsets of particularly high correlation (Figure 2C). These included a cluster of AZA metabolism genes encoding the ribonucleotide reductase regulatory subunits M1 and M2 (RRM1 and RRM2) (correlation cluster 2) and a cluster containing CBP-encoding gene CREBBP, EP300, and TET2 (correlation cluster 4).

Taken together, in a cohort of 36 MDS patient samples, we examined the expression of 50 genes and their correlation with each other.

Expression of a subset of genes correlates with key gene mutations

In the patient cohort, all known mutations frequently occurring in MDS were well represented (Figure 3A). Mutations co-occurred in variable combinations with low frequency. This encouraged us to determine the gene expression differences associated with a specific mutation (Figure 3B). For instance, in samples with mutation of the transcription factor runt-related transcription factor 1 (RUNX1), the genes encoding for the antiapoptotic member of the Bcl2 family BCL2L10, which is implicated in AZA resistance23 and RRM2, were down regulated (Figure 3C). Furthermore, in samples with serine/arginine-rich splicing factor 2 (SRSF2) mutation, BCL2L10 and the gene encoding for the ribosomal protein RRP1, were both down regulated (Figure 3D). Interestingly, BAZ1B, CHRAC1, RING1, SMARCA5 as well as the ribosome biogenesis/protein translation genes MALSU1, MRPS26, and POLR3H were all less expressed in samples with TET2 mutation (Figure 3E). Finally, mutation in the gene encoding for the tumor suppressor P53, TP53, correlated with lower expression of itself and MACROH2A1, while the AZA-transporter SLC28A3 and the AZA-target DNM1 were upregulated (Figure 3F).

Taken together, the expression levels of several genes significantly correlated with the mutational status of the samples.

Expression of different genes is associated with AZA response and overall survival

To approach the question whether the expression of any of the analyzed genes could be informative for predicting the patient’s response to AZA, we compared both responders (n = 14) and nonresponders (n = 22). Response was defined according to the international working group criteria (IWG 2006).24 As shown in Figure 4A, 7 out of all analyzed genes showed a significantly

| Table 1 Patient Characteristics and Mutation Frequency |
|-------------------------------------------------------|
| Characteristics | Numbers | Gene | Wildtype | Mutated | Na |
| Male              | 20 (56%) | ASXL1 | 21 (58.3%) | 15 (41.7%) |
| Female            | 16 (44%) | DNMT3A | 32 (88.9%) | 4 (11.1%) |
| Median age, y (range) | 70 (50-84) | EZH2 | 33 (91.7%) | 3 (8.3%) |
| RQMD              | 1 (2.8%) | FLT3.LM | 33 (91.7%) | 3 (8.3%) |
| CMML2             | 2 (5.6%) | IDH1 | 29 (80.6%) | 7 (19.4%) |
| RAEB1             | 4 (11.1%) | IDH2 | 32 (88.9%) | 4 (11.1%) |
| RAEB2             | 14 (38.9%) | KRAS | 34 (94.4%) | 2 (5.6%) |
| MDS/MPD           | 1 (2.8%) | MLL.PTD | 34 (94.4%) | 2 (5.6%) |
| sAML              | 11 (30.6%) | NRAS | 30 (83.3%) | 6 (16.7%) |
| tAML              | 3 (8.3%) | RUNX1 | 24 (66.7%) | 11 (30.6%) |
| Normal karyotype  | 15 (41.7%) | SFB31 | 32 (88.9%) | 4 (11.1%) |
| 5q (del)          | 7 (19.4%) | SRSF2 | 16 (44.4%) | 17 (47.2%) |
| chr7 (del)        | 6 (16.7%) | TET2 | 25 (69.4%) | 11 (30.6%) |
| chr8 (tris)       | 4 (11.1%) | TP53 | 30 (83.3%) | 6 (16.7%) |

CMML2 = chronic myelomonocytic leukemia type 2; del = deletion; MPD = myeloproliferative disorder; RAEB = refractory anemia with excess blasts; RQMD = refractory cytopenias with multilineage dysplasia; tris = trisomy.
Figure 2. Nanostring analysis in MDS patient samples to identify association of gene expression with main mutations, response to AZA treatment and overall survival. (A) Workflow of the Nanostring experiment. 36 bone marrow aspirates from MDS patients were obtained and DNA and RNA extracted. With the DNA, a gene mutation analysis was performed, while the RNA was used to determine the expression of 50 genes by Nanostring. The data analysis focused on main mutations, response to AZA treatment and overall survival. (B) Average mRNA counts (log10 scale) determined by Nanostring and normalized to housekeeping genes GUSB and TUBB. The Nanostring panel contained probes recognizing 15 selected screening hits, 16 CBP/p300 and protein synthesis genes (from Diesch et al.), 8 AZA metabolism genes, 6 MDS-related genes and 5 other genes. (C) Correlation matrix of normalized mRNA counts. Spearman correlation coefficients and the corresponding P values were calculated. red, negative correlation; blue, positive correlation; cross, not significant (P value > 0.01). Four main correlation clusters could be identified. AZA = azacitidine.
increased expression in patients that responded to AZA. These genes were \textit{UCK1}, \textit{CREBBP}, \textit{CHRAC1}, \textit{SMARCA5}, \textit{MALSU1}, \textit{ASXL1}, and \textit{TET2}. \textit{UCK1} encodes an AZA-activating enzyme and its positive association with response has been observed before.\textsuperscript{16}

Another way to analyze the expression data from patient samples is to correlate gene expression with patient survival. For this, the median expression of each gene was calculated, and the samples split between low (below median) and high (above median) expression. The 2 subgroups were compared based on the median overall survival (Table 2; Suppl. data S1). In Figure 4B, the genes whose low and high expression led to significant differences (\(P\) value \(\leq 0.05\)) in overall survival are shown.

Patients with lower \textit{MRTO4} expression had a median survival of 20 months compared to 12 months with higher \textit{MRTO4} expression. Similarly, patients with low \textit{NAA10} expression had a median survival of nearly 25 months, while patients with high expression survived only 8 months. Patients with low \textit{POLR1E} expression had a median survival of 20 months compared to 15 months for patients with high expression. In contrast, patients with low expression of \textit{NSUN3} survived only 11 months, while patients with high expression had a median survival of close to 25 months. In addition to the univariate analysis, we performed a multivariate analysis using the Cox model considering the 4 genes with significant difference in overall survival. The gene that remained statistically significant was \textit{NAA10} (hazard ratio [95% CI]): \(3.3 (1.5, 7.1), P = 0.003\).

Taken together, we identified several genes with significantly different expressions in responders versus nonresponders, as well as 4 genes for which the median overall survival differed depending on the expression level.

In conclusion, we determined the association of expression of 50 selected genes with AZA response in vitro together with mutational status, AZA response and survival in vivo. We identified a few potentially interesting genes warranting to be further evaluated for their potential as combinatorial drug targets or response-predicting biomarkers.
Figure 4. Expression of a subset of genes correlates with AZA response or overall survival. (A) mRNA read counts were normalized to the mean of 2 housekeeping genes and then to the average of all samples. Patients were divided into no-responders (n = 22) and responders (n = 14). Statistical analysis was performed using Student’s t-test. *P value ≤ 0.05. (B) Patients were divided into low (below median, blue) and high (above median, green) expression and the survival probability calculated. Only survival probabilities with significant differences (P value ≤ 0.05) are shown. Statistical analysis was performed using the Log-Rank (Mantel-Cox) test. AZA = azacitidine.
**DISCUSSION**

Primary and secondary resistances are the major limitations for treatment success of malignant diseases. This is also the case for MDS patients treated with AZA. Here we have taken a dual approach to identify candidate genes that might serve as response-predicting biomarkers or combinatorial drug targets. In addition, we found genes, whose expression is altered in the presence of specific recurrent mutations. By performing shRNA screening in the sAML cell line SKK-1, we identified genes whose knockdown affected sensitivity to AZA treatment in both directions. A gene whose knockdown caused resistance to AZA was ERCC2. The ERCC2 gene encodes for a protein important in the nucleotide excision repair pathway, which is involved in repairing different types of DNA damage. Its common polymorphism Lys751Gln leads to decreased activity and is significantly associated with breast, colorectal, pancreatic, bladder, lung, and hematological malignancies. This is in line with the here made observation that knockdown of ERCC2 led to increased AZA resistance. Moreover, in a recent study by Stopka et al., the closely related ERCC1 gene has been found to be mutated in AZA-resistant AML cell lines as well as in paired MDS samples from patients before and after development of AZA resistance. Thus, ERCC1/ERCC2 are interesting candidates and their involvement in AZA resistance should be further examined.

Furthermore, we have identified the genes FBXO11 and FLYWCH1, whose knockdown increased the resistance to AZA. FBXO11 encodes for the F-box only protein 11, which is part of a ubiquitination complex that indirectly impacts the differentiation of B-cells and plasma cells. In line with our results, it is thought to be a tumor suppressor in myeloid malignancies and the loss of FBXO11 expression correlates with the progression of MDS to sAML. FLYWCH1 encodes for an only recently characterized protein involved in WNT/beta-catenin signaling in AML. In particular, it is thought as a negative regulator of nuclear beta-catenin activity, and thus, has a possible tumor suppressor role, which is in line with our results.

Genes whose knockdown increases the sensitivity of cells to AZA are potential combinatorial drug targets. In our experiment, this included five genes encoding components of the ISWI chromatin remodeling. ISWI is an ATP-dependent complex implicated in nucleosome assembly, spacing and maturation, as well as DNA damage repair and chromatin cohesion. Specifically, we identified CHRAC1, BAZ1A, BAZ1B, SMARCA5, and POLE3 as genes whose knockdown caused a survival and growth disadvantage in the presence of AZA. SMARCA5 (also known as SNF2H) is one of the 2 possible ATPases of the ISWI complex. In many cell types, SMARCA5 is an essential gene and was shown to be required for embryonic development and fetal hematopoiesis. SMARCA5 is highly expressed in CD34+ AML cells and became downregulated after hematologic remission. While the screen was based on single copy integrations of individual hairpin cassettes, we have switched to multicopy integrations of selected hairpin cassettes for validation. Under these conditions, most likely leading to stronger gene suppression, the knockdown of SMARCA5 was not tolerated. However, the knockdown of other ISWI components such as CHRAC1, POLE3, and BAZ1A was tolerated and allowed us to validate their role in AZA sensitivity. At the present, it is unclear how ISWI affects AZA sensitivity, but an involvement in the repair of AZA-induced DNA damage is a valid hypothesis that warrants testing. As our and others’ data suggest, ISWI is an interesting candidate for therapeutic intervention in myeloid diseases. While directly targeting the ATPase SMARCA5 might not be feasible, it is worth to explore targeting other ISWI complex components as alternative strategy.

By analyzing the association between mutational status and gene expression in an MDS patient cohort, we could observe several interesting correlations. As the statistical power of our analysis is limited by the modest number of 36 patients, these correlations should be taken with caution and validated in further experiments. In RUNXI mutant samples, we saw a reduction of BCL2L10 expression in comparison to RUNXI wildtype samples. BCL2L10 is an antiapoptotic member of the BCL2 family and involved in chemo-resistance in various cancers. Specifically in MDS, high expression of BCL2L10 poses a valid hypothesis that warrants testing. As a result, we have identified the genes ERCC1, BAZ1A, and POLE3 as genes whose knockdown caused a survival and growth disadvantage in the presence of AZA.

*Table 2*

| Probe Name   | Median Survival—Low Expressed (mo) | Median Survival—High Expressed (mo) | P Value |
|--------------|-----------------------------------|------------------------------------|---------|
| ASXL1        | 14.50                             | 20.57                              | 0.180   |
| BAZ1A        | 17.63                             | 16.10                              | 0.433   |
| BAZ1B        | 15.67                             | 18.90                              | 0.513   |
| BCL2L10      | 17.63                             | 10.53                              | 0.479   |
| CDA          | 17.63                             | 15.67                              | 0.521   |
| CHRACT       | 15.17                             | 18.90                              | 0.477   |
| CREBBP       | 15.17                             | 18.90                              | 0.608   |
| DNM1         | 16.10                             | 23.70                              | 0.574   |
| DNM7A        | 15.17                             | 20.57                              | 0.909   |
| ERCC2        | 15.17                             | 24.50                              | 0.212   |
| FBXO11       | 15.17                             | 20.43                              | 0.464   |
| FLYWCH1      | 14.50                             | 20.57                              | 0.824   |
| HELS         | 17.63                             | 14.50                              | 0.976   |
| IDH1         | 15.67                             | 17.63                              | 0.760   |
| IDH2         | 17.63                             | 16.10                              | 0.624   |
| MACROH2A1    | 13.67                             | 23.70                              | 0.120   |
| MACROH2A2    | 18.90                             | 14.50                              | 0.330   |
| MALS1        | 12.74                             | 13.43                              | 0.441   |
| MLPL4        | 17.63                             | 16.10                              | 0.513   |
| MRPL52       | 15.17                             | 20.57                              | 0.495   |
| MRPS26       | 15.16                             | 23.70                              | 0.601   |
| MRT04        | 20.43                             | 11.77                              | 0.024   |
| NAA10        | 24.50                             | 7.70                               | 0.002   |
| NAA15        | 15.17                             | 20.57                              | 0.422   |
| NSUN3        | 10.53                             | 24.50                              | 0.024   |
| POL3         | 16.10                             | 17.63                              | 0.194   |
| POLR1A       | 14.50                             | 20.43                              | 0.585   |
| POLR1B       | 17.63                             | 11.77                              | 0.164   |
| POLR1C       | 18.90                             | 16.10                              | 0.377   |
| POLR1E       | 20.43                             | 14.50                              | 0.03    |
| POLR3D       | 15.17                             | 20.57                              | 0.729   |
| POLR3H       | 17.63                             | 16.10                              | 0.880   |
| PWP2B        | 15.17                             | 20.43                              | 0.782   |
| RING1        | 14.50                             | 24.50                              | 0.076   |
| RIOX2        | 15.17                             | 23.70                              | 0.577   |
| RRMI         | 20.57                             | 10.53                              | 0.425   |
| RRMI2        | 23.70                             | 10.53                              | 0.231   |
| RRP1         | 15.67                             | 20.57                              | 0.835   |
| RR9         | 17.63                             | 16.10                              | 0.315   |
| SLC28A3      | 23.70                             | 11.77                              | 0.102   |
| SLC29A1      | 18.90                             | 15.67                              | 0.926   |
| SMARCA5      | 15.17                             | 23.70                              | 0.647   |
| TET2         | 14.50                             | 20.43                              | 0.308   |
| TPS3         | 14.50                             | 24.50                              | 0.308   |
| UCK1         | 15.17                             | 20.43                              | 0.381   |
| UCK2         | 18.90                             | 16.10                              | 0.241   |
| EP300        | 15.67                             | 18.90                              | 0.671   |
between TET2 mutation and the ISWI complex. To date, no direct or indirect mechanism of interaction has been proposed, but considering our results this could be the starting point of a mechanistic study of the relation between TET2 and the ISWI complex. In TP53 mutant samples, SLC28A3 expression was upregulated. SLC28A3 is a pyrimidine and purine nucleoside transporter responsible for the cellular uptake of AZA and decitabine, and thus inhibition of SLC28A3 reduces the effect of AZA and decitabine.41,42 Interestingly, SLC28A3 has been shown to be a synthetically lethal gene for TP53.43 Two genes are synthetically lethal if the disruption of either of them does not result in cell death, whereas disruption of both genes (either through gene mutation or targeted therapy) does selectively kill the cells.44 Hence, in TP53 mutant patients, targeting SLC28A3 might be of particular interest, although the effect on AZA response would need to be considered.

Comparing the gene expression to AZA response and overall survival we identified different sets of genes. In AZA responders, UCK1, CREBBP, CHRAC1, SMARCA5, MALSU1, ASXL1, and TET2 had a significantly increased expression compared to nonresponders. UCK1 is an enzyme essential for the metabolism of AZA and its integration into RNA and DNA.45 Its upregulation in responders compared to nonresponders is in accordance with what has been previously reported.16 This reinforces the idea of using UCK1 expression as a predictive biomarker of the response to AZA treatment. For SMARCA5, the higher expression in responders contrasted with what we would have expected from the functional studies in which knockdown-induced low levels favored response.

The overall survival analysis is an important indicator for treatment success. We did not observe any significant differences in overall survival for patients with high versus low expression of UCK1 and the other response-associated genes possibly due to the limited statistical power of our cohort size. However, we identified 4 genes, including NAA10 and NSUN3, whose expression led to significant changes. Overexpression of N-terminal acetyltransferases, particularly NAA10, is seen in various tumor types and correlates with a poor prognosis.45 Our observation that high expression of NAA10 leads to a significant lower overall survival, is in line with these reports. NSUN3 is a mitochondrial tRNA methyltransferase and mediates methylation of mitochondrial tRNA(Met) at cytosine 34.46 Furthermore, it has recently been shown to interact directly with DNMT2 and hnRNPK leading to the recruitment of RNA polymerase II at nascent RNA and the formation of an AZA-sensitive chromatin structure.47 If and how different expression levels of NSUN3 affect the formation of distinct chromatin complexes has not been examined, but the here demonstrated correlation between NSUN3 expression and overall survival in MDS patients does lead to the hypothesis that higher expression might favor the formation of AZA-sensitive chromatin structure and thereby better survival.

Taken together, here, we identified several genes implicated in AZA sensitivity in vitro as well as AZA response, mutational status and overall survival in vivo. These 2 gene sets were largely nonoverlapping indicating that response modulators in vitro are not necessarily response predictors in vivo. A small patient cohort size as well as general differences between in vitro treatments and the complex treatment responses seen in patients, in which the tumor environment and immune system play important roles, might explain these discrepancies. Future studies will be needed to further evaluate the genes of the first set as potential combinatorial drug targets and the genes of second set as response-predicting biomarkers.

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AUTHOR CONTRIBUTIONS

Conceptualization: MB; Formal analysis: MMLP, JD; Funding acquisition: MB; Investigation: MMLP, JD; Methodology: ZJ; Resources: TH, KSG, AK; Visualization: JD; Writing – original draft: JD, MMLP, MB; Writing – review and editing: JD, MMLP, MB.

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