HemaSphere

EHA EUROPEAN HEMATOLOGY ASSOCIATION

Article
Open Access

Different Gene Sets Are Associated With Azacitidine Response In Vitro Versus in Myelodysplastic Syndrome Patients

Marguerite-Marie Le Pannérer[1], Jeannine Diesch[1,2], Raquel Casquero[1], Michael Maher[1], Olga Garcia[3,4], Torsten Haferlach[5], Johannes Zuber[6,7], Andrea Kündgen[8], Katharina S. Götze[9], Marcus Buschbeck[1,2]

Correspondence: Marcus Buschbeck (mbuschbeck@carrerasresearch.org).

ABSTRACT

Myelodysplastic syndromes (MDS) are a heterogeneous group of hematopoietic disorders characterized by dysplasia, ineffective hematopoiesis, and predisposition to secondary acute myeloid leukemias (sAML). Azacitidine (AZA) is the standard care for high-risk MDS patients not eligible for allogenic bone marrow transplantation. However, only half of the patients respond to AZA and eventually all patients relapse. Response-predicting biomarkers and combinatorial drugs targets enhancing therapy response and its duration are needed. Here, we have taken a dual approach. First, we have evaluated genes encoding chromatin regulators for their capacity to modulate AZA response. We were able to validate several genes, whose genetic inhibition affected the cellular AZA response, including 4 genes encoding components of Imitation SWItch chromatin remodeling complex pointing toward a specific function and co-vulnerability. Second, we have used a classical cohort analysis approach measuring the expression of a gene panel in bone marrow samples from 36 MDS patients subsequently receiving AZA. The gene panel included the identified AZA modulators, genes known to be involved in AZA metabolism and previously identified candidate modulators. In addition to confirming a number of previously made observations, we were able to identify several new associations, such as *NSUN3* that correlated with increased overall survival. Taken together, we have identified a number of genes associated with AZA response in vitro and in patients. These groups of genes are largely nonoverlapping suggesting that different gene sets need to be exploited for the development of combinatorial drug targets and response-predicting biomarkers.

INTRODUCTION

Myelodysplastic syndrome (MDS) is a disease of dysregulated clonal hematopoiesis with defective cell maturation leading to one or more cytopenias.[1] 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.[2–6] 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[7] or of a single "tipping point" mutation.[8] 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).[9]

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.[10] 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 al[11]). However, half of the patients do not respond to treatment with azanucleosides and the other half eventually acquires resistance leading to relapse.[12,13] Numerous studies have explored mechanisms of resistance to azanucleosides or biomarkers to predict response to treatment. For example, Tet methylcytosine dioxygenase 2

[1]*Cancer and Leukaemia Epigenetics and Biology Program, Josep Carreras Leukaemia Research Institute (IJC), Badalona, Spain*
[2]*Program for Predictive and Personalized Medicine of Cancer, Germans Trias i Pujol Research Institute (PMPPC-IGTP), Badalona, Spain*
[3]*Clinical and experimental Hematology Program, Josep Carreras Leukaemia Research Institute (IJC), Badalona, Spain*
[4]*Hematology Department, ICO Badalona Hospital Germans Trias I Pujol, Badalona, Spain*
[5]*Munich Leukemia Laboratory, Munich, Germany*
[6]*Research Institute of Molecular Pathology, Vienna BioCenter, Vienna, Austria*
[7]*Medical University of Vienna, Vienna BioCenter, Vienna, Austria*
[8]*Department of Hematology, Oncology, and Clinical Immunology, Heinrich-Heine-University Hospital Duesseldorf, Germany*
[9]*Department of Medicine III, Klinikum rechts der Isar, Technische Universität München, Germany*
M-MLP and JD have contributed equally to this work.
Supplemental digital content is available for this article.

HemaSphere (2022) 6:11(e792).
http://dx.doi.org/10.1097/HS9.0000000000000792.
Received: July 25, 2022 / Accepted: September 22, 2022

(*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 pRRL-UCOE SFFV-GFP-miRE-PGK-Puro (cSGEP) backbone were described previously.[19] For the production of lentiviral particles, we used packaging plasmids psPax2 (Addgene #12260) and pCMV-VSV-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 Drexler and been characterized in detail.[20] MOLM-13 has a *FLT3* internal tandem duplication (FLT3-ITD), an MLL-AF9 fusion and mutations in *CBL*, *KMT2A*, and *NF1* genes.[20] SKK-1 cells are available from the original authors.[21] Cells were maintained in RPMI 1640 (Gibco, Thermo Scientific, Waltham, MA) supplemented with 10% of FBS, 1% penicillin-streptomycin and 1% L-Gutamine (Gibco) at 37°C in 5% CO_2. HEK293T (ATCC # CRL-11268) were obtained from ATCC and cultured in Dulbecco's modified Eagle's media (DMEM, Gibco) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% L-Glutamine (Gibco) at 37°C in 5% CO_2. Cells were authenticated and passaged for <6 months.

Viral transduction and generation of stable cell lines

Cells were transduced using standard lentiviral transduction procedures. In brief, viral supernatants generated in transfected HEK293T cells were mixed with 8 µg/mL polybrene (Sigma-Aldrich, Sant Luis, MO) and used to infect 2×10^6 cells/well in 6-well plates by centrifuging at 500*g* for 30 minutes at 37°C. This procedure was repeated once before starting selection with 1 µg/mL puromycin 48 hours after the first infection.

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 1µg/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, 7.3×10^7 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 nanostring analysis

Bone marrow samples from patients were obtained from the Munich Leukemia Laboratory (MLL) and originated from the cohort described in Kündgen et al.[15] Samples were stored in 300 µL RLT buffer + ß-Mercaptoethanol at −80°C. RNA extraction was performed using the Qiagen RNeasy kit according to instructions. 100 ng in 5µL final volume (20 ng/µL) were prepared and 8 µL of mastermix (Reporter 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, 555 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.

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[21] and harbors mutations in splicing factor *U2AF1* and the chromatin regulator *BCOR*.[20] To identify genes, whose knockdown would be increasing the response to AZA, we have titrated AZA down to concentrations barely affecting the viability of SKK-1 cells by themselves. Based on the results shown in Figure 1A, we chose an AZA concentration of 0.075 µM and extended the treatment period to 21 days (Figure 1B). This approach contrasts with our previous screen performed for 14 days treatment with 0.25 µM AZA, which, apart from CBP, primarily identified genes whose knockdown caused resistance.[19] To functionally test genes, we used a previously described RNA interference-based library targeting 912 chromatin and transcriptional regulators with 8 different shRNAs per gene.[19] SKK-1 cells were

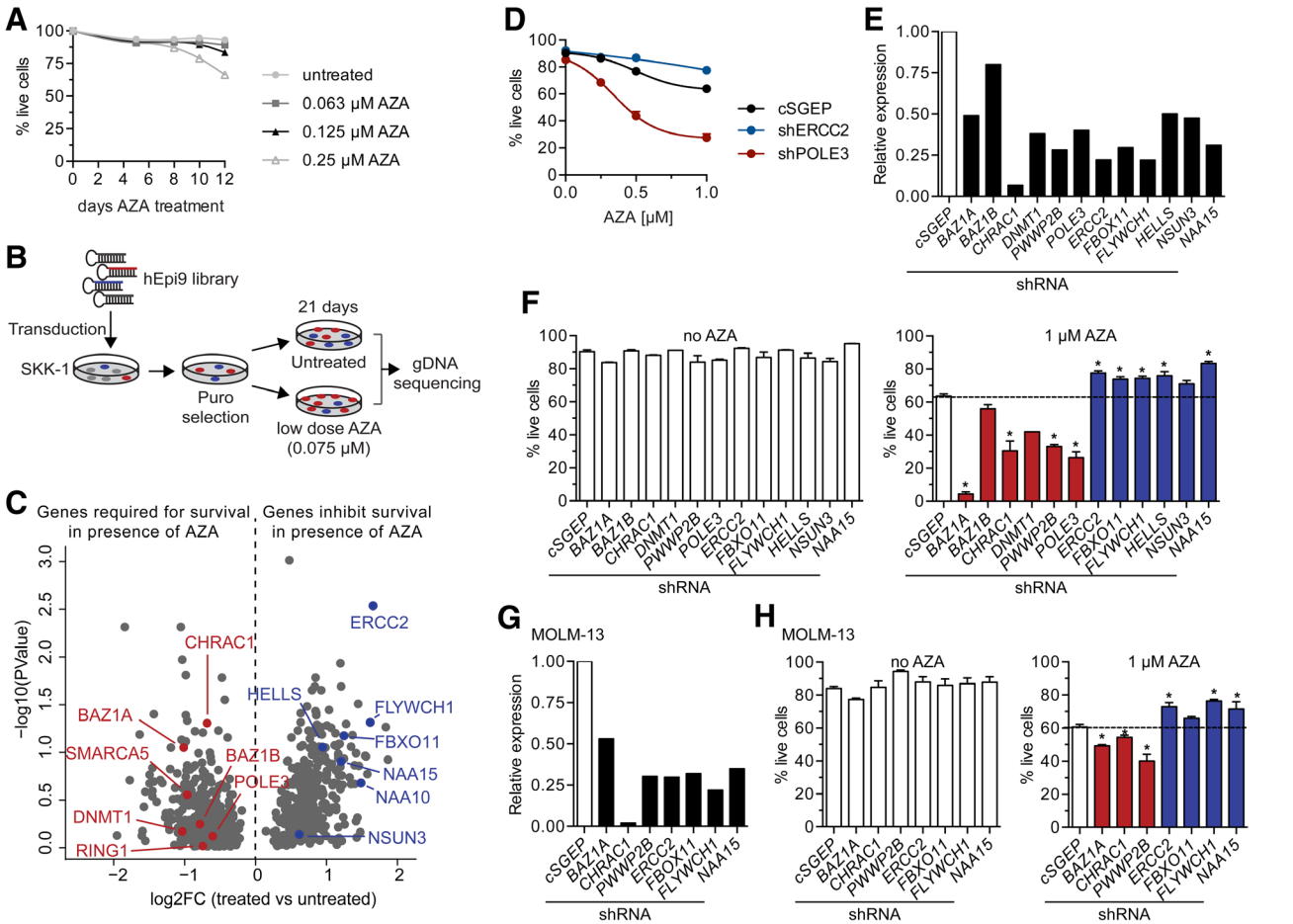


Figure 1. Chromatin regulators affecting AZA sensitivity are identified by a loss-of-function shRNA screen. (A) Titration of AZA in SKK-1 cells to determine concentration used in shRNA screen. (B) shRNA screen workflow. SKK-1 cells were infected with hEpi9 library, puromycin selected, and treated with 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.

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 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

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,[22] 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 25 AZA treatment cycles with an average of 6.8 cycles. A cycle is defined as 75 mg/m^2 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 concentration.[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 resistance,[11] 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 erythrocytic 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 resistance[23] 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 processing 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 *DNMT1* 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%)	
RCMD	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%)	1 (2.8%)
Normal karyotype	15 (41.7%)	SF3B1	32 (88.9%)	4 (11.1%)	
5q (del)	7 (19.4%)	SRSF2	16 (44.4%)	17 (47.2%)	3 (8.3%)
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; RCMD = refractory cytopenias with multilineage dysplasia; tris = trisomy.

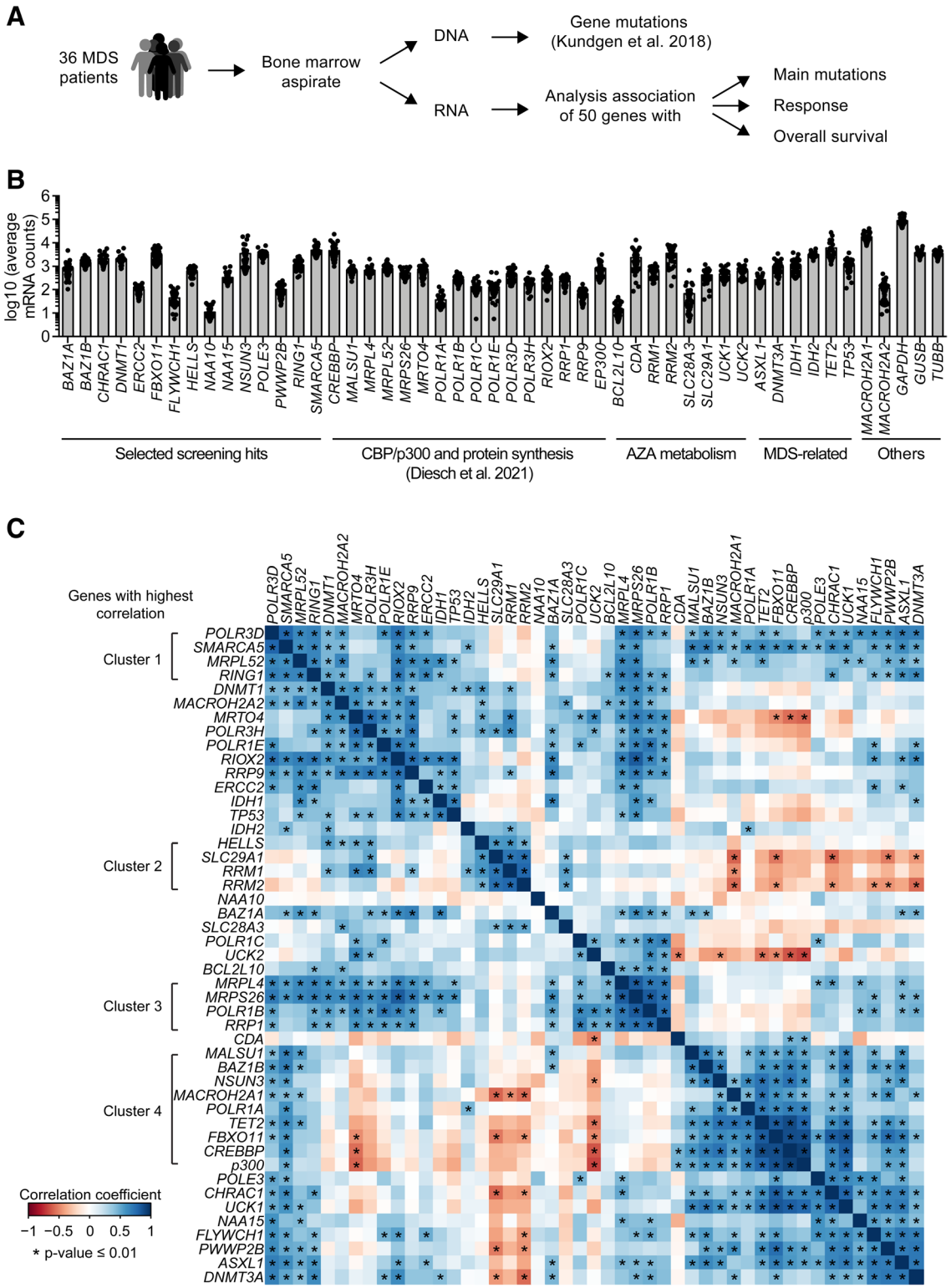


Figure 2. Nanostring analysis in MDS patient samples to identify association of gene expression with main mutations, response to AZA treatment and overall survival. (A) Workflow of the Nanostring experiment. 36 bone marrow aspirates from MDS patients were obtained and DNA and RNA extracted. With the DNA, a gene mutation analysis was performed,[15] 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[10]), 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.

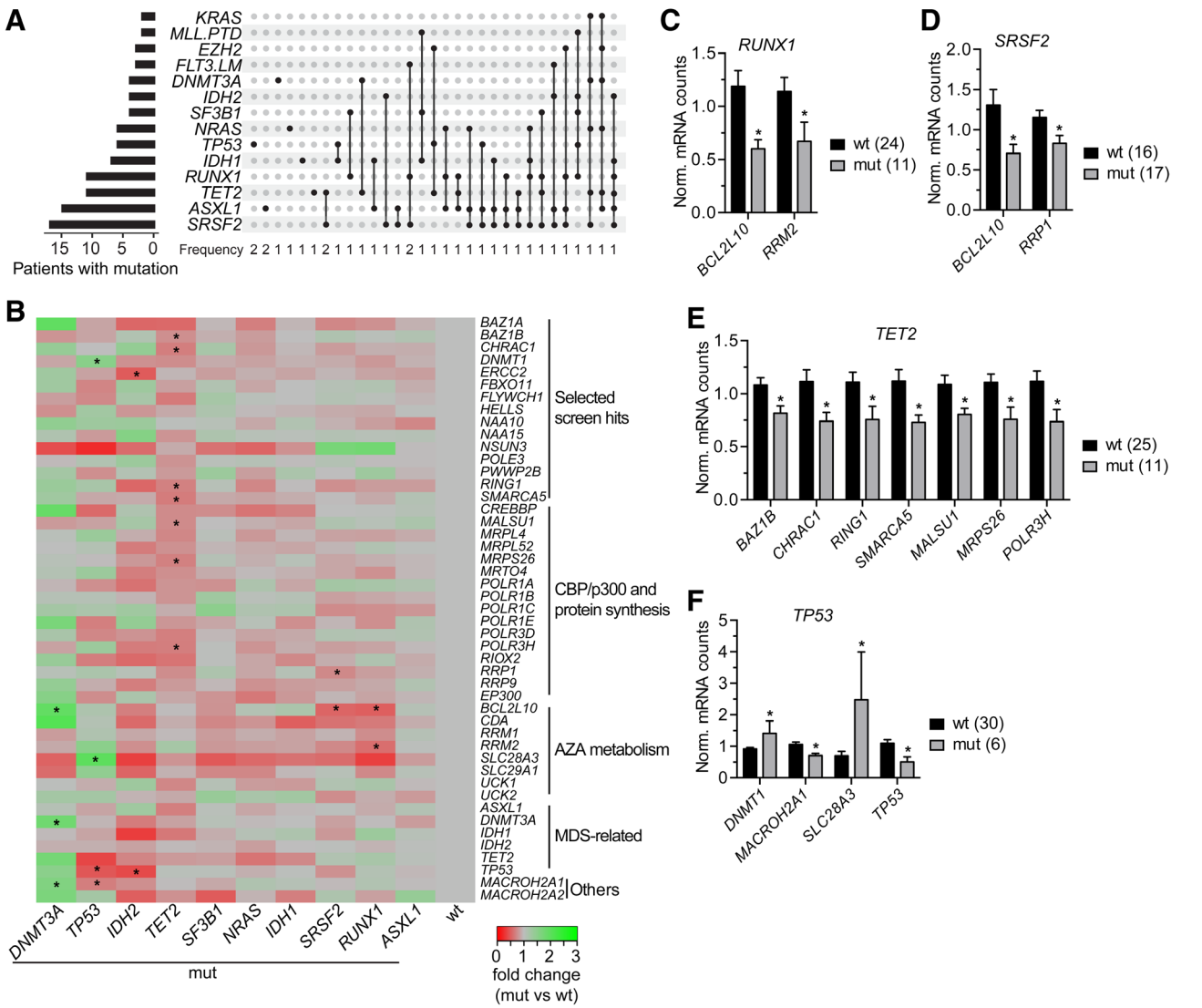


Figure 3. Expression of a subset of genes correlates with key gene mutations. (A) UpSetR plot of mutational status in patient cohort. All identified mutations and mutation combinations were present with a frequency of maximum 2 and thus are equally represented. (B) Relative fold change in mRNA levels of mutant versus wildtype (wt) samples. red, decrease; gray, no change; green, increase. (C–H) mRNA read counts were normalized to the mean of 2 housekeeping genes and then to the average of all samples. Only genes significantly different in mutant versus wt samples are displayed in (C) 11 patients with RUNX1 mutations, (D) 17 patients with SRSF2 mutations, (E) 11 patients with TET2 mutations, and (F) 6 patients with TP53 mutations. (C–H) Statistical analysis was performed using Student's t-test. *P value ≤ 0.05.

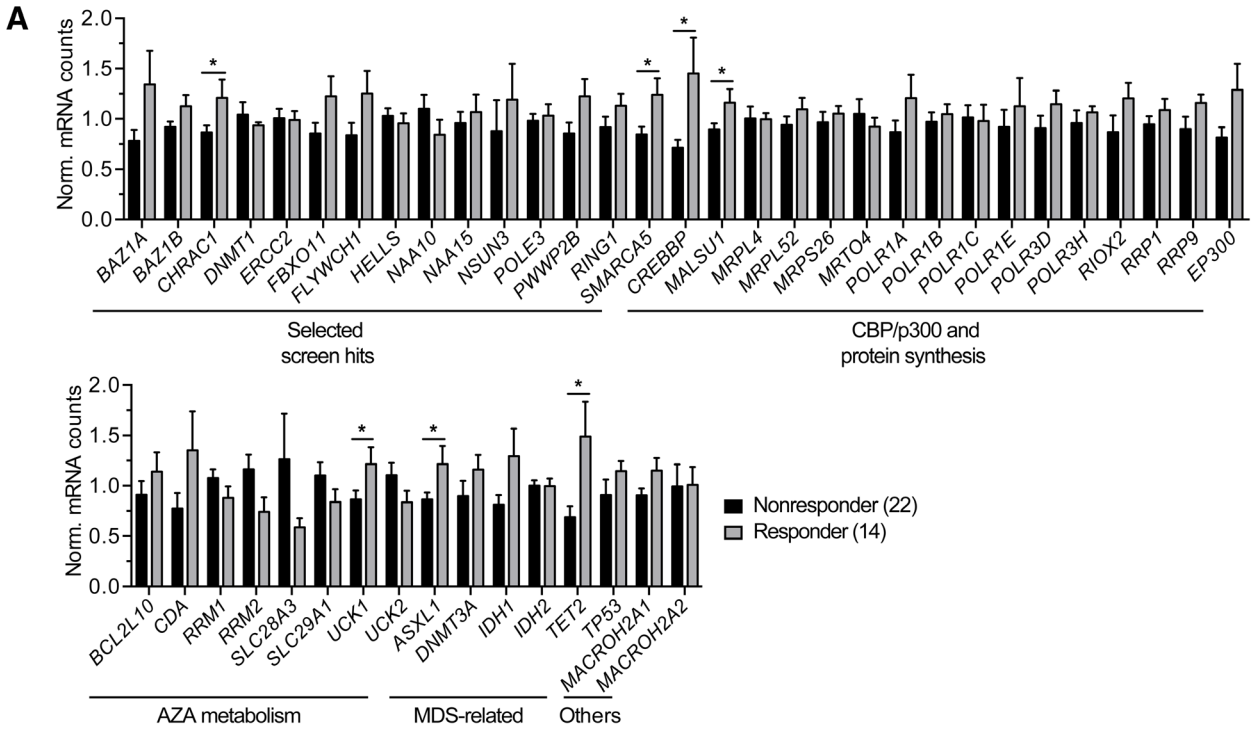
increased expression in patients that responded to AZA. These genes were *UCK1*, *CREBBP*, *CHRAC1*, *SMARCA5*, *MALSU1*, *ASXL1*, and *TET2*. *UCK1* encodes an AZA-activating enzyme and its positive association with response has been observed before.[16]

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 ≤ 0.05) in overall survival are shown.

Patients with lower *MRTO4* expression had a median survival of 20 months compared to 12 months with higher *MRTO4* expression. Similarly, patients with low *NAA10* expression had a median survival of nearly 25 months, while patients with high expression survived only 8 months. Patients with low *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 *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 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.



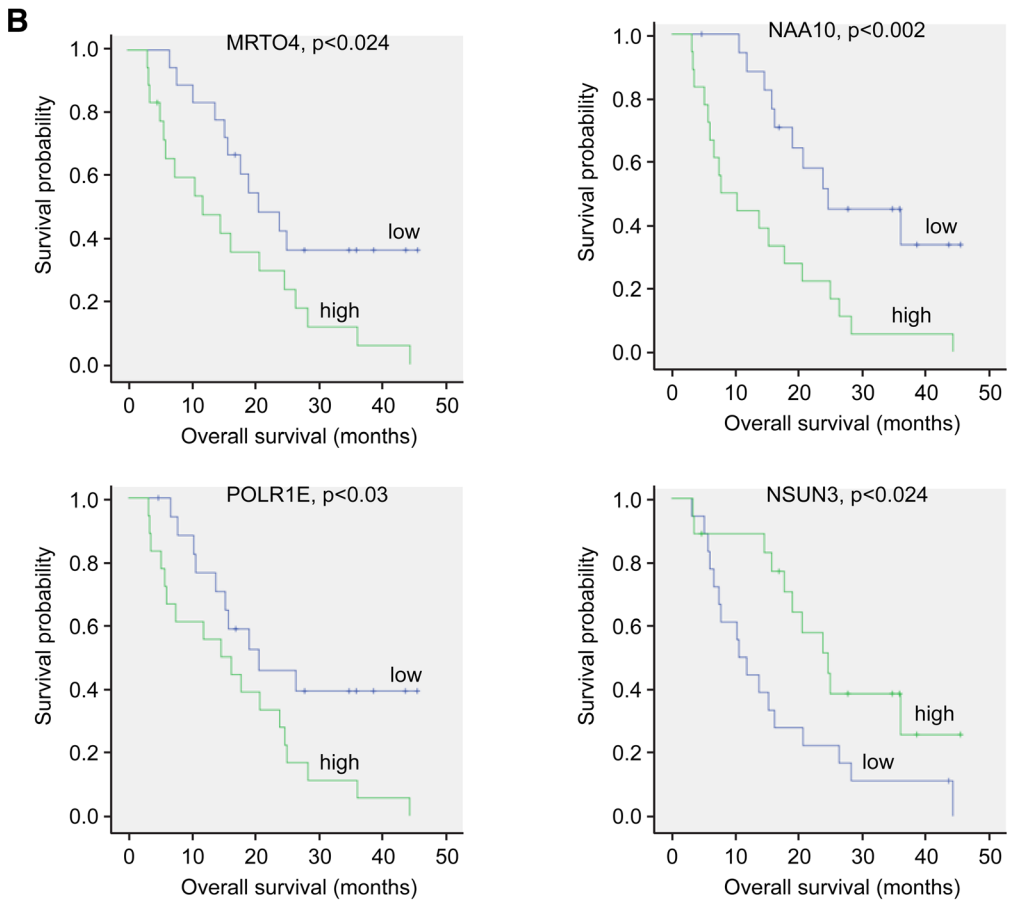


Figure 4. Expression of a subset of genes correlates with AZA response or overall survival. (A) mRNA read counts were normalized to the mean of 2 housekeeping genes and then to the average of all samples. Patients were divided into no-responders (n = 22) and responders (n = 14). Statistical analysis 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.

Table 2

Analysis of Patient Survival Based on Gene Expression

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
CHRAC1	15.17	18.90	0.477
CREBBP	15.17	18.90	0.608
DNMT1	16.10	23.70	0.574
DNMT3A	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
HELLS	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
MALSU1	12.74	13.43	0.441
MRPL4	17.63	16.10	0.513
MRPL52	15.17	20.57	0.459
MRPS26	15.16	23.70	0.601
MRTO4	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
POLE3	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
PWWP2B	15.17	20.43	0.782
RING1	14.50	24.50	0.076
RIOX2	15.17	23.70	0.577
RRM1	20.57	10.53	0.425
RRM2	23.70	10.53	0.231
RRP1	15.67	20.57	0.835
RRP9	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
TP53	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

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.[25] Its common polymorphism Lys751Gln leads to decreased activity and is significantly associated with breast, colorectal, pancreatic, bladder, lung, and hematological malignancies.[26–29] 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,[30] 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 knockdowns 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.[31] *FLYWCH1* encodes for an only recently characterized protein involved in WNT/ß-catenin signaling in AML. In particular, it is thought as a negative regulator of nuclear ß-catenin activity, and thus, has a possible tumor suppressor role,[32] 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 chromatid cohesion.[33] 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.[34–36] *SMARCA5* is highly expressed in CD34+ AML cells and became downregulated after hematologic remission.[37,38] 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 *RUNX1* mutant samples, we saw a reduction of *BCL2L10* expression in comparison to *RUNX1* wildtype samples. BCL2L10 is an antiapoptotic member of the BCL2 family and involved in chemo-resistance in various cancers.[39] Specifically in MDS, a high percentage of BCL2L10 positive bone marrow cells correlated with lower response rate and shorter overall survival.[23] Targeting BCL2L10 with ABT-737 has been shown to be particularly promising in BCL2L10-expressing AZA-resistant leukemic cells.[40] In *TET2* mutant samples, we saw that genes involved in ribosome biogenesis/protein translation were downregulated, as well as various genes encoding components of the ISWI complex. Some prior articles loosely established a relation

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.[11] 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 a 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.

ACKNOWLEDGEMENTS

We would like to thank all members of the Buschbeck lab, the RESPONSE network (ISCIII/PIE16/00011), Blanca Xicoy, and Lurdes Zamora for valuable discussions. We thank Marco Fernandez for advice and training in flow cytometry and all other staff of IJC and IGTP core facilities for excellent support.

AUTHOR CONTRIBUTIONS

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

DISCLOSURES

KSG has received support for independent research from Celgene Corp and MB from CellCentric Ltd. All the other authors have no conflicts of interest to disclose.

SOURCES OF FUNDING

This research project was supported by the grant PIE16/00011 RESPONSE (to MB), the European Research Council ERC-StG-336860 (to JZ), the grant Juan de la Cierva—Formación FJCI-2014-22983 (to JD), the grant Sara Borrell CD17/00084 (to JD), the SFB 1243 DFG (to KSG) the Austrian Science Fund SFB-F4710 (to JZ) and the Deutsche José Carreras Leukämie Stiftung DJCLS 14R/2018 (to KSG and MB) and the FPI fellowship BES-2016-077251 (to MMLP). Research in the Buschbeck lab was further supported by the following grants: the Marie Skłodowska Curie Training network "INTERCEPT-MDS" H2020-MSCA-ITN-2020-953407 (to MB and KG); PID2021-126907NB-I00 from FEDER/ Ministerio de Ciencia y Innovación - Agencia Estatal de Investigación (to MB); AGAUR 2017-SGR-305 (to MB) and Fundació La Marató de TV3 257/C/2019 (to MB). Research at the IJC was supported by the "La Caixa" Foundation, the Fundació Internacional Josep Carreras, Celgene Spain and the CERCA Programme/Generalitat de Catalunya.

REFERENCES

1. Sperling AS, Gibson CJ, Ebert BL. The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. *Nat Rev Cancer*. 2017;17:5–19.
2. Papaemmanuil E, Gerstung M, Malcovati L, et al. Clinical and biological implications of driver mutations in myelodysplastic syndromes. *Blood*. 2013;122:3616–27; quiz 3699.
3. Haferlach T, Nagata Y, Grossmann V, et al. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia*. 2014;28:241–247.
4. Bersanelli M, Travaglino E, Meggendorfer M, et al. Classification and personalized prognostic assessment on the basis of clinical and genomic features in myelodysplastic syndromes. *J Clin Oncol*. 2021;39:1223–1233.
5. Adema V, Palomo L, Toma A, et al. Distinct mutational pattern of myelodysplastic syndromes with and without 5q– treated with lenalidomide. *Br J Haematol*. 2020;189:e133–e137.
6. Khoury JD, Solary E, Abla O, et al. The 5th edition of the World Health Organization classification of haematolymphoid tumours: myeloid and histiocytic/dendritic neoplasms. *Leukemia*. 2022;36:1703–1719.
7. Lindsley RC, Mar BG, Mazzola E, et al. Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood*. 2015;125:1367–1376.
8. Shukron O, Vainstein V, Kündgen A, et al. Analyzing transformation of myelodysplastic syndrome to secondary acute myeloid leukemia using a large patient database. *Am J Hematol*. 2012;87:853–860.
9. Awada H, Thapa B, Visconte V. The genomics of myelodysplastic syndromes: origins of disease evolution, biological pathways, and prognostic implications. *Cells*. 2020;9:2512.
10. Saber W, Horowitz MM. Transplantation for myelodysplastic syndromes: who, when, and which conditioning regimens. *Hematology*. 2016;2016:478–484.
11. Diesch J, Zwick A, Garz A-k, et al. A clinical-molecular update on azanucleoside-based therapy for the treatment of hematologic cancers. *Clin Epigen*. 2016;2016:1–11.
12. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol*. 2009;10:223–232.
13. Garcia-Manero G, Fenaux P. Hypomethylating agents and other novel strategies in myelodysplastic syndromes. *J Clin Oncol*. 2011;29:516–523.

14. Bejar R, Lord A, Stevenson K, et al. TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. *Blood*. 2014;124:2705–2712.
15. Kuendgen A, Müller-Thomas C, Lauseker M, et al. Efficacy of azacitidine is independent of molecular and clinical characteristics—an analysis of 128 patients with myelodysplastic syndromes or acute myeloid leukemia and a review of the literature. *Oncotarget*. 2018;9:27882–27894.
16. Valencia A, Masala E, Rossi A, et al. Expression of nucleoside-metabolizing enzymes in myelodysplastic syndromes and modulation of response to azacitidine. *Leukemia*. 2014;28:621–628.
17. Estey EH. Acute myeloid leukemia: 2021 update on risk-stratification and management. *Am J Hematol*. 2020;95:1368–1398.
18. Sallman DA, DeZern AE, Garcia-Manero G, et al. Eprenetapopt (APR-246) and Azacitidine in TP53-Mutant Myelodysplastic Syndromes. *J Clin Oncol*. 2021;39:1584–1594.
19. Diesch J, Le Pannérer MM, Winkler R, et al. Inhibition of CBP synergizes with the RNA-dependent mechanisms of Azacitidine by limiting protein synthesis. *Nat Commun*. 2021;12:6060.
20. Palau A, Mallo M, Palomo L, et al. Immunophenotypic, cytogenetic, and mutational characterization of cell lines derived from myelodysplastic syndrome patients after progression to acute myeloid leukemia. *Genes Chromosomes Cancer* 2017;56:243–252.
21. Matsuoka H, Murayama T, Koizumi T, et al. Establishment of a human myeloid cell line with trisomy 8 derived from overt leukemia following myelodysplastic syndrome. *Haematologica*. 2005;90:981–982.
22. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–951.
23. Vidal V, Robert G, Goursaud L, et al. BCL2L10 positive cells in bone marrow are an independent prognostic factor of azacitidine outcome in myelodysplastic syndrome and acute myeloid leukemia. *Oncotarget*. 2017;8:47103–47109.
24. Cheson BD, Greenberg PL, Bennett JM, et al. Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia. *Blood*. 2006;108:419–425.
25. Wood RD, Mitchell M, Sgouros J, et al. Human DNA repair genes. *Science*. 2001;291:1284–1289.
26. Taylor EM, Broughton BC, Botta E, et al. Xeroderma pigmentosum and trichothiodystrophy are associated with different mutations in the XPD (ERCC2) repair/transcription gene. *Proc Natl Acad Sci USA*. 1997;94:8658–8663.
27. Artac M, Bozcuk H, Pehlivan S, et al. The value of XPD and XRCC1 genotype polymorphisms to predict clinical outcome in metastatic colorectal carcinoma patients with irinotecan-based regimens. *J Cancer Res Clin Oncol*. 2010;136:803–809.
28. Chacko P, Rajan B, Joseph T, et al. Polymorphisms in DNA repair gene XRCC1 and increased genetic susceptibility to breast cancer. *Breast Cancer Res Treat*. 2005;89:15–21.
29. Liu D, Wu D, Li H, et al. The effect of XPD/ERCC2 Lys751Gln polymorphism on acute leukemia risk: A systematic review and meta-analysis. *Gene*. 2014;538:209–216.
30. Minařík L, Pimková K, Kokavec J, et al. Analysis of 5-azacytidine resistance models reveals a set of targetable pathways. *Cells*. 2022;11:223.
31. Schieber M, Marinaccio C, Bolanos LC, et al. FBXO11 is a candidate tumor suppressor in the leukemic transformation of myelodysplastic syndrome. *Blood Cancer J*. 2020;10:98.
32. Almars A, Chondrou PS, Onyido EK, et al. Increased FLYWCH1 expression is negatively correlated with Wnt/β-catenin target gene expression in acute myeloid leukemia cells. *Int J Mol Sci*. 2019;20:2739.
33. Erdel F, Rippe K. Chromatin remodelling in mammalian cells by ISWI-type complexes--where, when and why? *FEBS J*. 2011;278:3608–3618.
34. Stopka T, Skoultchi AI. The ISWI ATPase Snf2h is required for early mouse development. *Proc Natl Acad Sci USA*. 2003;100:14097–14102.
35. Kokavec J, Zikmund T, Savvulidi F, et al. The ISWI ATPase smarca5 (Snf2h) is required for proliferation and differentiation of hematopoietic stem and progenitor cells. *Stem Cells*. 2017;35:1614–1623.
36. Zikmund T, Kokavec J, Turkova T, et al. ISWI ATPase smarca5 regulates differentiation of thymocytes undergoing β-selection. *J Immunol (Baltimore, Md: 1950)*. 2019;202:3434–3446.
37. Stopka T, Zakova D, Fuchs O, et al. Chromatin remodeling gene SMARCA5 is dysregulated in primitive hematopoietic cells of acute leukemia. *Leukemia*. 2000;14:1247–1252.
38. Zikmund T, Paszekova H, Kokavec J, et al. Loss of ISWI ATPase SMARCA5 (SNF2H) in acute myeloid leukemia cells inhibits proliferation and chromatid cohesion. *Int J Mol Sci*. 2020;21:2073.
39. Yasui K, Mihara S, Zhao C, et al. Alteration in copy numbers of genes as a mechanism for acquired drug resistance. *Cancer Res*. 2004;64:1403–1410.
40. Cluzeau T, Robert G, Karsenti J-M, et al. BCL2L10 (Bcl-B) is associated with resistance to azacitidine (AZA) in MDS and AML, and is a possible therapeutic target in AZA resistant patients. *Blood*. 2012;120:701–701.
41. Ueda K, Hosokawa M, Iwakawa S. Cellular uptake of decitabine by equilibrative nucleoside transporters in HCT116 Cells. *Biol Pharm Bull*. 2015;38:1113–1119.
42. Hummel-Eisenbeiss J, Hascher A, Hals PA, et al. The role of human equilibrative nucleoside transporter 1 on the cellular transport of the DNA methyltransferase inhibitors 5-azacytidine and CP-4200 in human leukemia cells. *Mol Pharmacol*. 2013;84:438–450.
43. Wang X, Sun Q. TP53 mutations, expression and interaction networks in human cancers. *Oncotarget*. 2017;8:624–643.
44. Kaelin WG, Jr. The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer*. 2005;5:689–698.
45. Koufaris C, Kirmizis A. N-terminal acetyltransferases are cancer-essential genes prevalently upregulated in tumours. *Cancers*. 2020;12:2631.
46. Nakano S, Suzuki T, Kawarada L, et al. NSUN3 methylase initiates 5-formylcytidine biogenesis in human mitochondrial tRNA(Met). *Nat Chem Biol*. 2016;12:546–551.
47. Cheng JX, Chen L, Li Y, et al. RNA cytosine methylation and methyltransferases mediate chromatin organization and 5-azacytidine response and resistance in leukaemia. *Nat Commun*. 2018;9:1163.