ASXL1 mutations are associated with a response to alvocidib and 5-azacytidine combination in myelodysplastic neoplasms

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Abstract

Inhibitors of anti-apoptotic BCL-2 family proteins in combination with chemotherapy and hypomethylating agents (HMA) are promising therapeutic approaches in acute myeloid leukemia (AML) and high-risk myelodysplastic syndromes (MDS). Alvocidib, a cyclin-dependent kinase 9 (CDK9) inhibitor and indirect transcriptional repressor of the anti-apoptotic factor MCL-1, has previously shown clinical activity in AML. Availability of biomarkers for response to the alvocidib + 5-azacytidine (5-AZA) could also extend the rationale of this treatment concept to high-risk MDS. In this study, we performed a comprehensive *in vitro* assessment of alvocidib and 5-AZA effects in N=45 high-risk MDS patients. Our data revealed additive cytotoxic effects of the combination treatment. Mutational profiling of MDS samples identified *ASXL1* mutations as predictors of response. Further, increased response rates were associated with higher gene expression of the pro-apoptotic factor NOXA in *ASXL1*-mutated samples. The higher sensitivity of *ASXL1* mutant cells to the combination treatment was confirmed *in vivo* in *ASXL1*^{Y588X} transgenic mice. Overall, our study demonstrated augmented activity for the alvocidib + 5-AZA combination in higher-risk MDS and identified *ASXL1* mutations as a biomarker of response for potential stratification studies.

Introduction

Myelodysplastic syndromes (MDS) are a group of heterogeneous clonal diseases of hematopoietic stem cells leading to cytopenia and an increased risk of developing secondary acute myeloid leukemia (sAML). Treatment with the hypomethylating agent (HMA) 5-azacytidine (5-AZA) is standard-of-care for higher-risk MDS patients that extends overall survival.[1] However, only approximately 50% of patients initially respond to the therapy and the development of secondary resistance is almost certain.[1,2] Therefore, combination of 5-AZA with drugs that facilitate differentiation or apoptosis of high-risk MDS hematopoietic stem cells and progenitors (HSPC) is a potential therapeutic approach.

In high-risk MDS, anti-apoptotic members of the BCL-2 family show a higher expression level compared to low-risk MDS and healthy bone marrow (BM).[3] This leads to decreased rates of apoptosis or resistance to apoptosis.[4] Inhibitors of anti-apoptotic BCL-2 family members in com-

bination with 5-AZA have recently shown potent activity against AML and high-risk MDS. It was demonstrated that venetoclax, a highly selective BCL-2 inhibitor, and 5-AZA act synergistically to eliminate AML cells *in vitro* and display synergistic antitumor activity in AML patients *in vivo*.[5,6] Besides, selective inhibitors of the anti-apoptotic protein MCL-1 alone or in combination with venetoclax showed broad cytotoxicity against primary low- and high-risk MDS cells.[7]

Alvocidib (Flavopiridol) is a broad inhibitor of cyclin-dependent kinases (CDK), including CDK1, CDK2, CDK4, CDK6, CDK7 and CDK9.[8] Previous studies indicated that the cytotoxic effects of alvocidib are primarily mediated via inhibition of CDK7 and CDK9.[8,9] Mechanistically, CDK7 and CDK9 inhibition by alvocidib repressed transcriptional elongation mediated by RNA polymerase II resulting in the downregulation of its target genes.[8,10] One of the primary proposed alvocidib target genes is the RNA polymerase II-dependent gene *MCL-1* due to the fast turnover of its RNA and protein.[11] In MCL-1-dependent hematologic malignancies, downregulation of MCL-1 expression through inhibition of CDK9 was reported to induce apoptosis of leukemic blasts.[10,12] Alvocidib alone or in combination therapies has previously shown clinical activity in hematologic malignancies.[13,14] Encouraging anti-leukemic effects of alvocidib in combination with cytarabine/mitoxantrone or cytarabine/daunorubicin were reported in phase I and II studies of AML.[14,15] Recently, the effects of alvocidib in combination with HMA decitabine or 5-AZA had been investigated in a phase Ib/II study in patients with high-risk MDS (*clinicaltrials gov. Identifier: NCT03593915*). However, this study was terminated for reasons of company strategy and not powered to identify biomarkers associated with the response to alvocidib and 5-AZA + alvocidib combination. Therefore, the aim of our study was to characterize potential biomarkers of response to alvocidib + 5-AZA combination treatment in MDS, which was not previously assessed in preclinical models or clinical trials.

We report that the increased sensitivity of primary MDS samples to the alvocidib or alvocidib + 5-AZA treatment *in vitro* is associated with the presence of *ASXL1* mutations. These results were further confirmed in *Asxl1*^{Y588X} transgenic mice. Higher cytotoxic effects of alvocidib in *ASXL1*-mutant samples correlated with elevated expression of MCL-1 interacting pro-apoptotic factor NOXA. These novel insights suggest that the stratification of patients based on *ASXL1* mutational status may be beneficial for the selection of particularly eligible patients into clinical trials with this drug combination.

Methods

Routine protocols are described in detail in the *Online Supplementary Appendix*.

Myelodysplastic syndromes patient and healthy control samples

Samples of MDS patients (N=45) were obtained from diagnostic BM aspirations. Patient characteristics are presented in Table 1. Individual patient data, including patient ID are presented in the *Online Supplementary Table S1*. Hematopoietic cells from age-matched hematologically healthy controls (HC, N=15), median 69 years (range, 47-92) were obtained from bone specimen after femur endoprosthesis surgery. All patients and HC provided written informed consent and all interventions were performed in accordance with the Declaration of Helsinki. Human material in this study was used following institutional review board approval by the Medical Ethics Committee II of the Medical Faculty Mannheim, University of Heidelberg, Germany and Columbia University's institutional review board-approved tissue repository (IRB-AAAF2693).

In vivo experimental procedures

The development of leukemia in the *Asxl1*^{Y588X} transgenic (Tg) mice used in this study takes about 12 months.[16] For the tumor transfer assay, 3×10^6 splenic cells from 12-month-old C57BL/6 *Asxl*^{Y588X} Tg mice with myeloid leukemia were injected into sublethally irradiated (6.0 Gy) CD45.1$^+$ B6.SJL-Ptprca Pepcb/BoyJ wild-type (WT) recipients through tail veins. Seven weeks after the tumor

Table 1. Clinical parameters of patients.

Parameters	N=45
Median age in years (range)	73 (43-90)
Sex, N	
Male	34
Female	11
WHO-subtype 2016, N	
MDS-MLD	6
MDS-RS-MLD	1
MDS-EB1	10
MDS-EB2	20
MDS/MPN-U	3
CMML-2	1
AML-MRC	2
sAML	2
IPSS-R, N	
Intermediate	5
High	23
Very high	12
N/A (sAML, AML-MRC)	4
CPSS, N	
High	1

WHO: World Health Organization; MDS-MLD: myelodysplastic syndromes with multilineage dysplasia; MDS-RS-MLD: MDS with ring sideroblasts and MLD; MDS-EB: MDS with excess blasts; MDS/MPN-U: myelodysplastic/myeloproliferative neoplasm, unclassifiable; CMML: chronic myelomonocytic leukemia; N/A: not applicable; sAML: secondary acute myeloid leukemia; AML-MRC: AML with myelodysplasia-related changes; IPSS-R: Revised International Prognostic Scoring System; CPSS: CMML-specific prognostic scoring system.

transfer, mice were treated with 1 mg/kg 5-AZA intraperitoneally (i.p.) on days 1-3 (once daily, qd) every other week. Alvocidib was administered i.p. at a dose of 2.5 mg/kg or 5 mg/kg on day 5 (once weekly, qw) in the weeks when 5-AZA was applied. Mice were sacrificed 11 weeks after the tumor transfer and the percentage of CD45.1$^+$ and CD45.2$^+$ cells in the BM was assessed by flow cytometric analysis. All animal experiments were done in accordance with the guidelines of the University of Texas Health San Antonio Animal Care and Use Facility.

In xenotransplantation experiments, 1x10^6 MDS-L cells[17] were injected intravenously (i.v.) into busulfan-conditioned NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg (CMV-IL3,CSF2,KITLG)1Eav/MloySzJ (NSGS) mice as previously described.[18] Following engraftment (on day 14), 5-AZA (0.5 mg/kg on days 1-4), alvocidib (5 mg/kg on day 5), or the combination of both were administered i.p. for the duration of the experiment. In order to determine the effects of drugs on leukemic burden, blood samples were collected from all mice 21 and 50 days post-transplantation and percentage of human CD45$^+$ MDS-L cells in mouse blood was assessed using flow cytometry. Animals were bred and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility of Cincinnati Children's Hospital Medical Center. Xenograft experiments were approved by the Cincinnati Children's Hospital IACUC (protocol #2020-0073). All experiments conform to the regulatory standards of the Institutional Animal Care and Use Committee (IACUC) and adhere to IACUC-approved protocols.

Hematopoietic stem and progenitor cell culture and cell viability assays

Human hematopoietic stem and progenitor cells (HSPC) were expanded for 5 days in StemSpan™ SFEM II medium (Stem Cell Technologies, 09655) containing StemSpan™ Myeloid Expansion Supplement (Stem Cell Technologies, 02693) before treatment with alvocidib for 24 hours (h), 5-AZA for 48 h or sequential combination of 5-AZA for 48 h followed by alvocidib for 24 h. CellTiter-Glo (CTG) and Annexin V apoptosis assays were used as cell viability readouts and described in details in the *Online Supplementary Appendix*.

Results

The treatment combination of alvocidib + 5-AZA exerts additive cytotoxic effects on myelodysplastic syndromes cells *in vitro* and *in vivo*

In order to determine alvocidib and 5-AZA concentrations for drug combination studies, dose-response experiments using CD34$^+$ cells isolated from the BM of high-risk MDS patients (N=16) and HC (N=12) were performed. CD34$^+$ cells were expanded for 5 days in StemSpan™ SFEM II medium containing StemSpan™ Myeloid Expansion Supplement and subjected to CTG cell viability assay after 24 h of incubation with alvocidib or 48 h of incubation with 5-AZA. Of note, the sensitivity of MDS cells to alvocidib and especially 5-AZA was more heterogeneous as compared to HC (Figure 1A; *Online Supplementary Figure S1*). In order to assess the effects of the alvocidib + 5-AZA combination on the viability of CD34$^+$ MDS HSPC, median IC30 concentrations for MDS samples were tested in CTG assays. The cells were sequentially treated with 5-AZA for 48 h followed by alvocidib treatment for 24 h in line with the sequential dosing schedule of Ib/II MDS study (*clinicaltrials gov. Identifier: NCT03593915*) and our previous data.[19] The combination treatment had a statistically significant additive cytotoxic effect on CD34$^+$ cells from high-risk MDS patients (Figure 1B), which was also observed in apoptosis assays (Figure 1C). Of note, the cytotoxic effects of alvocidib + 5-AZA were more pronounced against MDS cells as compared to healthy HSPC (Figure 1D). Similarly, the percentage of late apoptotic and dead cells was significantly higher in MDS samples as compared to HC (Figure 1D). In order to check, whether the potential presence of clonal hematopoiesis of indeterminate potential (CHIP) mutations in HC sensitizes CD34$^+$ cells to alvocidib + 5-AZA treatment, we performed myeloid deep panel sequencing of N=9 available HC MNC samples. There were no detectable mutations in eight of nine HC samples, whereas one HC had an acquired DNMT3A mutation (variant allele frequency [VAF] =33%), which was confirmed by Sanger sequencing (*Online Supplementary Table S2*; *Online Supplementary Figure S2*). This mutated sample (labeled in dark red color in Figure 1F, G) was not an outlier in HC control group based on its sensitivity to the combination treatment (ROUT outlier test, Q=1%).

We next tested the activity of alvocidib + 5-AZA in NSGS mice engrafted with MDS-L cells. Similar to our *in vitro* studies, the combination treatment resulted in an accelerated reduction of the leukemic burden compared to single substances (Figure 1E). In order to address the issue of the substance combination toxicity *in vivo*, we collected data about body weight changes during the treatment. We also assessed the impact of single agents and their combination on the hematological parameters in NSGS mice transplanted with MDS-L cells. Our data showed that both single agents and their combination did not affect mouse body weights during the 37 days of treatment (*Online Supplementary Figure S3A*). Both 5-AZA and alvocidib moderately decreased white blood cell (WBC) counts with marginal statistical significance (*Online Supplementary Figure S3B*). However, there was no aggravated negative effect of the combination treatment on the WBC levels as compared to the single substances. As expected, 5-AZA decreased red blood cell (RBC) and hemoglobin values in treated mice. However, alvocidib did not show toxic effects on erythroid cells. Moreover, toxic

effects of 5-AZA were not exacerbated by alvocidib (*Online Supplementary Figure S3C, D*). Alvocidib insignificantly increased platelet levels in treated mice. However, these were not further increased after combination treatment (*Online Supplementary Figure S3E*). In summary, *in vivo* data showed an overall favorable toxicity profile of alvo-

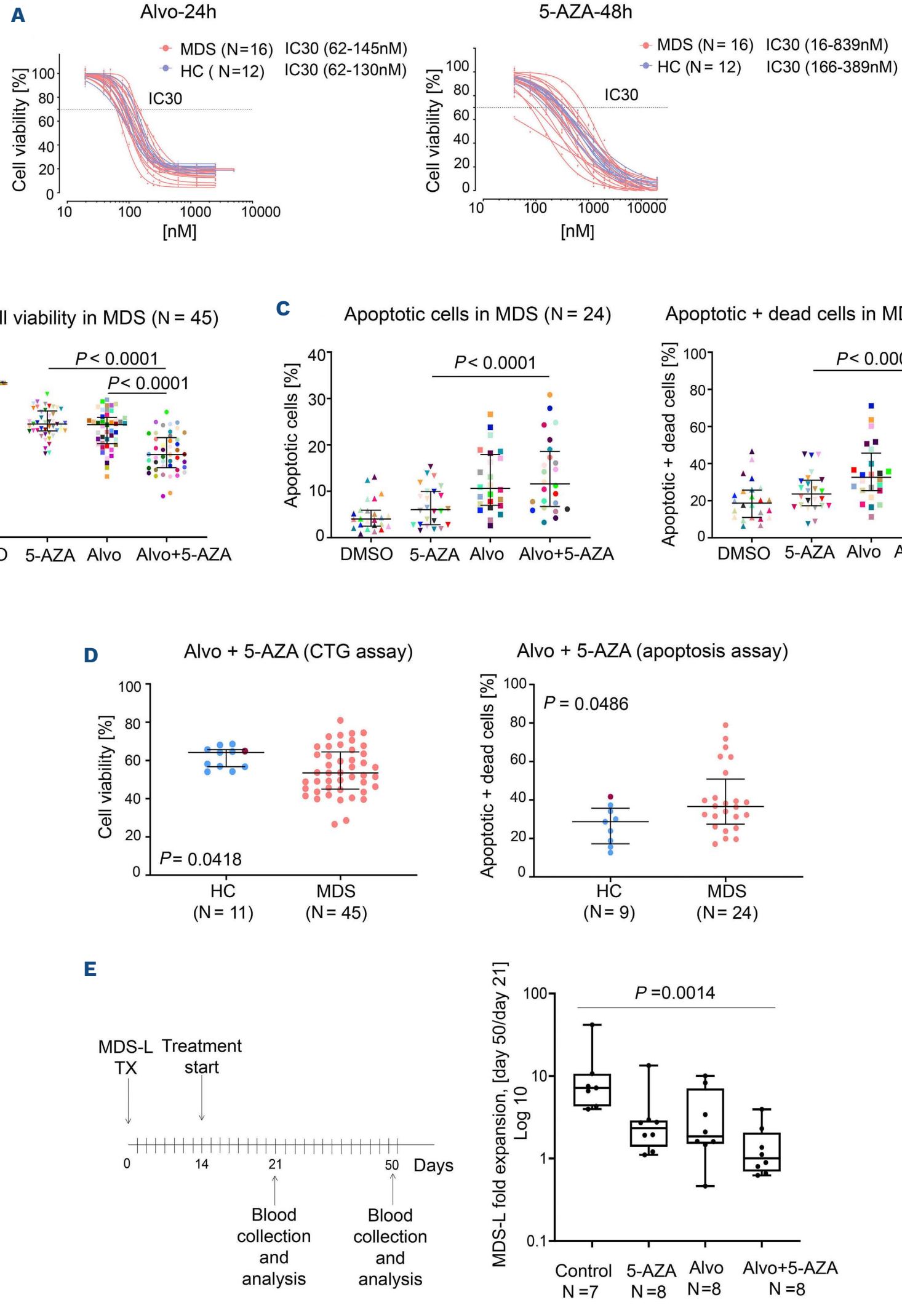

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Figure 1. The combination of alvocidib and 5-AZA exhibited an additive effect on the cell viability in human high-risk myelodysplastic syndromes samples. (A) Dose response curves were generated after alvocidib (Alvo) treatment for 24 hours (h) (left panel) and 5-azacytidine (5-AZA) treatment for 48 h (right panel) for N=16 myelodysplastic syndromes (MDS) patients and N=12 hematologically healthy controls (HC). Cell viability was measured using CTG assay. (B) Cell viability (CTG assay) was measured in N=45 MDS patient samples based on mean cell viability (IC30) concentrations of both drugs; median ± interquartile range (IQR), Friedman test with Dunn's multiple comparisons. (C) The percentages of apoptotic cells (left panel) and apoptotic + dead cells (right panel) were measured using Annexin V assay in N=24 MDS patient samples; median ± IQR; Friedman test with Dunn's multiple comparisons. (D) Cell viability (CTG assay, left panel) and percentages of apoptotic + dead cells (right panel) were compared after alvocidib + 5-AZA treatment in N=45 MDS patients *versus* N=11 HC; median ± IQR, Mann-Whitney U test. HC sample with DNMT3A W305fs frameshift mutation is labeled in dark red color. (E) MDS-L xenotransplantation experimental design and fold change in MDS-L expansion in blood between day 50 and day 21 of treatment of NSGS mice injected with MDS-L cells. Expansion fold change = % MDS-L cells at day 50 / % MDS-L cells at day 21. Expansion fold changes are presented as log10 transformed values. Box plots show medians ± IQR; whiskers indicate minimum and maximum; Kruskal-Wallis test with Dunn's multiple comparisons. TX: xenotransplantation; DMSO: dimethyl sulfoxide.

cidib + 5-AZA combination in mice.

ASXL1 mutations are associated with increased sensitivity to alvocidib and alvocidib + 5-AZA combination treatment in high-risk myelodysplastic syndromes

Since responses to the alvocidib + 5-AZA combination were markedly heterogeneous among MDS samples, we aimed to identify biomarkers predictive of higher sensitivity to the single substances or the combination of both. First, we correlated the response with the clinical parameters of the MDS patients. There was no significant association between patient survival and response to alvocidib or alvocidib + 5-AZA *ex vivo* treatment (*Online Supplementary Figure S4*). Responses to alvocidib and the combination treatment were not associated with patient age, sex, BM blasts, previous therapy, karyotype, IPSS-R, or hemoglobin levels (*Online Supplementary Figure S5*).

Next, we assessed whether heterogeneity in response to alvocidib and combination treatment could be explained by the differences in the MCL-1 dependency of MDS samples. We first compared sensitivity of *in vitro* expanded CD34$^+$ cells from N=9 high-risk MDS patients to MCL-1, BCL-2 and BCL-xL mimetics and found the most pronounced responses to highly specific MCL-1 mimetic S-63845 compared to BCL-2 and BCL-xL mimetics in eight of nine analyzed samples. This result further suggested MCL-1 inhibition as a plausible target in high-risk MDS (*Online Supplementary Figures S6, S7* and *S8*). However, when we defined responders and non-responders based on the mean cell viability (IC30) in the CTG assays, both of the groups were sensitive to MCL-1 mimetic (*Online Supplementary Figure S8*). In order to determine the association of MCL-1 dependency with alvocidib and alvocidib + 5-AZA response, BM MNC of MDS patients were incubated with T-MS1 peptide, a highly selective MCL-1 antagonist.[20] In MCL-1-dependent samples, this results in mitochondrial depolarization and apoptotic priming, which can be detected using a cationic dye that specifically accumulates in polarized mitochondria. Correlation of MCL-1 dependency with cell viability after alvocidib and combination treatment assessed in CTG assay revealed a reversed association between these parameters, suggesting that MCL-1-dependent samples were more sensitive to the alvocidib and combination treatment. The association was more pronounced in the combination treatment (Spearman r=-0.37, *P*=0.1119), but did not reach significance (*Online Supplementary Figure S9*). Overall, the results of apoptosis assay with BH3 mimetics and MCL-1 dependency assay did not predict the response of high-risk MDS samples to alvocidib and alvocidib + 5-AZA treatments. Therefore, to identify other potential biomarkers of response to alvocidib + 5-AZA, we performed myeloid panel sequencing of the most frequently mutated genes in MDS (N=67 genes) in BM MNC patient samples and associated the presence of somatic mutations with the treatment sensitivity. Forty-four of 45 patients (98%) carried mutations in at least one or more of these genes (Figure 2A). *ASXL1* (39% of patients), *TET2* (32%) and *RUNX1* (32%) were the most frequently mutated genes in the studied cohort (Figure 2A). When the patient samples were distributed into responder and non-responder groups based on the mean cell viability (IC30) in the CTG assays, the frequency of several mutations, including *ASXL1*, *EZH2*, *TET2*, *RUNX1*, *ZRSR2* and *STAG2* was higher in responder groups in both alvocidib and alvocidib + 5-AZA treatments arms (Figure 2B, C). We also associated the presence of mutations with cell viability after alvocidib and alvocidib + 5-AZA treatment as a continuous variable (Figure 3A, B). Higher sensitivity to alvocidib treatment was found in MDS samples with *ASXL1* (*P*=0.0100), *ZRSR2* (*P*=0.0035), *EZH2* (*P*=0.0020), *TET2* (*P*=0.0252) and *STAG2* (*P*=0.0104) mutations (Figure 3A). Interestingly, *SF3B1*, but not *SRSF2* mutations were associated with reduced sensitivity to alvocidib treatment (*Online Supplementary Figure S10*). Higher sensitivity to the combination treatment was associated with the presence of mutations in *ASXL1* (*P*=0.0037), *ZRSR2* (*P*=0.0068) and *EZH2* (*P*=0.0193) genes (Figure 3B). Of note, the presence of these mutations did not sensitize MDS samples to 5-AZA treatment alone (Figure 3C). In order to assess the effect of potential confounders, multivariable analysis was performed with sex, age, IPSS-R, *ASXL1*, *ZRSR2*, *TET2*, *RUNX1*, *EZH2* and *STAG2* mutations as co-variables. Only *ASXL1* mutations were found to be independently associated with

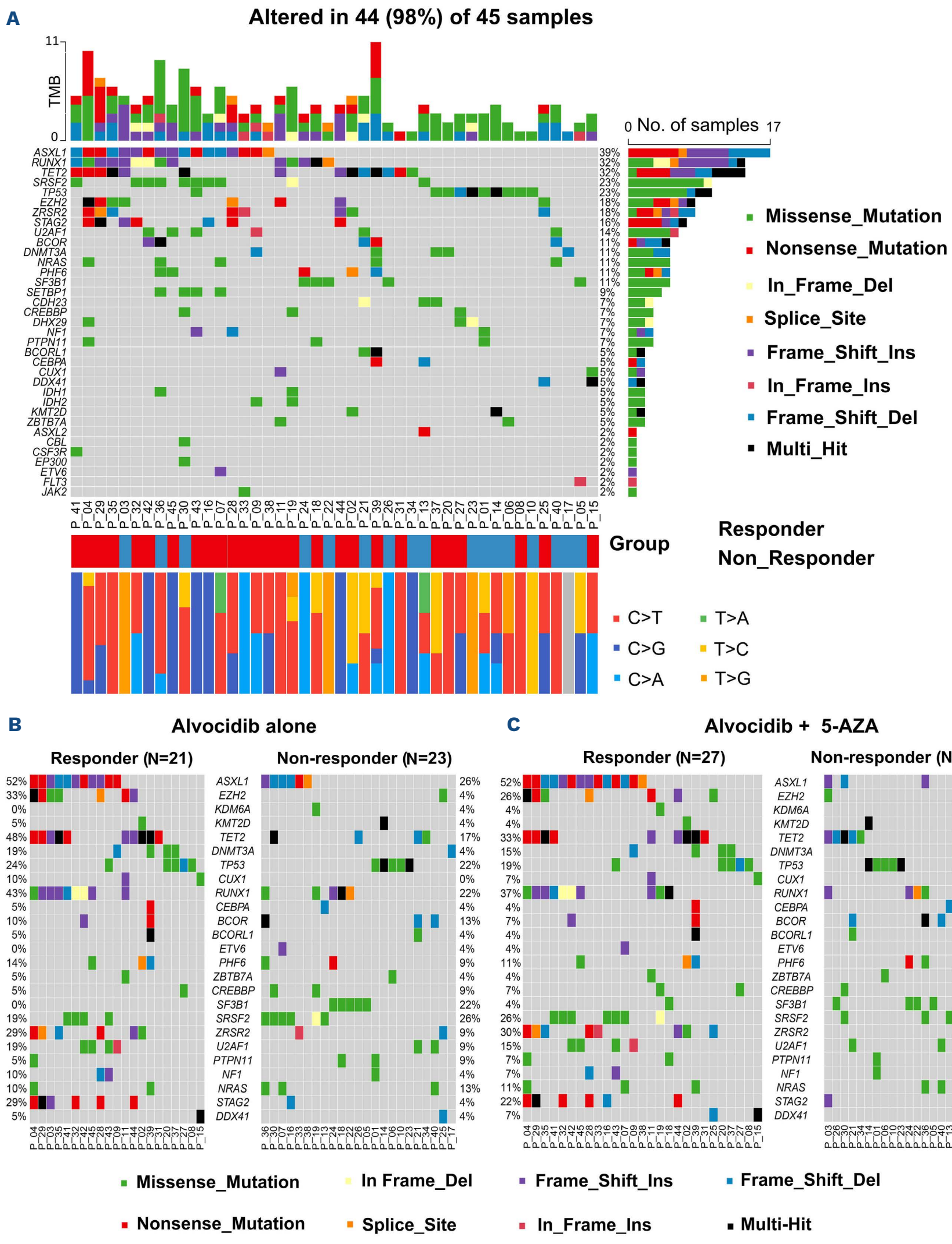
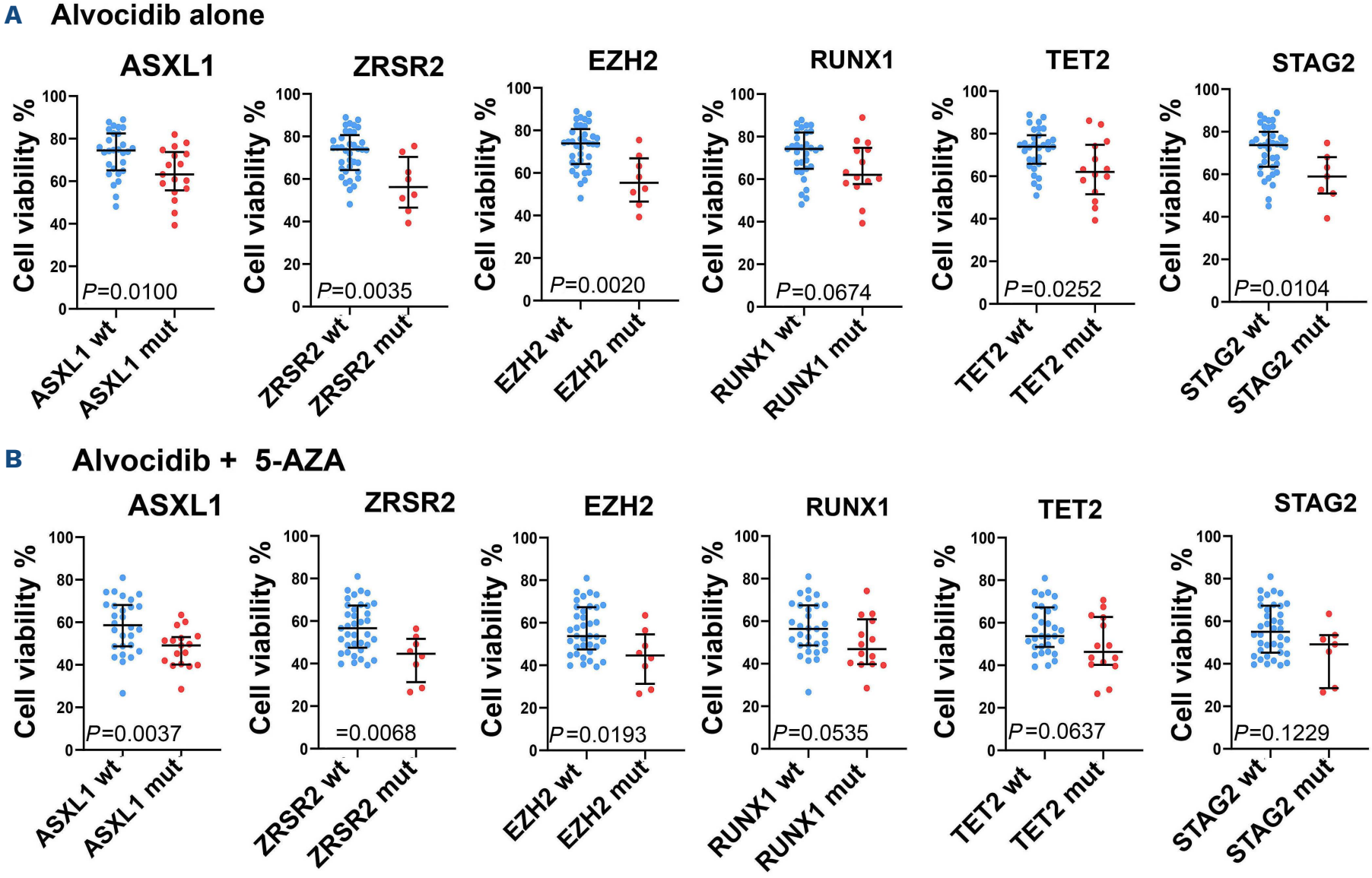


Figure 2. Mutational profiles in alvocidib and alvocidib + 5-AZA treated samples. (A) Bone marrow (BM) mononuclear cells (MNC) of myelodysplastic syndromes (MDS) patients (N=40) were subjected to myeloid panel deep sequencing. The mutational data for other N=5 MDS samples were obtained from the medical records. The mutations in MDS-associated genes were found in N=44 patients and presented as oncoplot. (B, C) MDS patients (N=44) were distributed into responder and non-responder groups based on the mean cell viability after alvocidib treatment (B) and alvocidib + 5-azacytidine (5-AZA) treatment (C) in CTG assay. Individual mutations for responders *versus* non-responders are presented as co-oncoplots. Del: deletion; Ins: insertion.

higher response to alvocidib + 5-AZA treatment, showing relative risk 0.058 with 95% confidence interval: 0.004-0.815 (P=0.035; Tables 2 and 3; Figure 3D). Therefore, in the subsequent experiments we focused on the role of *ASXL1* mutations in the alvocidib + 5-AZA sensitivity.

The *Asxl1*^{588X} mutation confers sensitivity to alvocidib and alvocidib + 5-AZA combination treatment in a murine myelodysplastic syndromes model

In order to study the association between specific mutations and sensitivity of MDS cells to alvocidib and combination treatment *in vivo*, we utilized a recently established Vav1 promoter-driven *Asxl1*^{Y588X} Tg mouse model expressing the analogous protein product of the mutant *ASXL1*^{Y591X}, frequently seen in human patients.[16] In order to check the sensitivity of BM cells from WT and *Asxl1*^{Y588X}Tg mice to alvocidib treatment, whole BM cells were treated with several concentrations of alvocidib and 5-AZA for 8 hours (h) and subjected to cell viability assessment using CTG assay (Figure 4A). Cytotoxic effects of alvocidib were more pronounced in *Asxl1*^{Y588X}Tg cells as compared to WT cells in all tested concentrations. The effects of 5-AZA were comparable in WT and *Asxl1*^{Y588X}Tg cells. Higher sensitivity to alvocidib resulted in higher cytotoxicity of alvocidib + 5-AZA combination for *Asxl1*^{Y588X} cells (Figure 4A). Next, we asked whether alvocidib + 5-AZA treatment preferentially targeted malignant over healthy hematopoiesis *in vivo*. For this, WT mice carrying CD45.1 alleles were reconstituted with 3×10^6 splenic cells from 12-month-old *Asxl1*^{Y588X} Tg leukemic mice carrying the CD45.2 allele. Seven weeks after reconstitution with leukemic cells, mice were treated with 5-AZA and alvocidib + 5-AZA according to the treatment schedule shown in Figure 4B. Flow cytometry assessment of BM before and after treatment revealed a statistically significant decrease of CD45.2$^+$ cells in the BM of mice treated with the alvocidib + 5-AZA combination (P=0.0156). This effect did not reach significance in mice treated with 5-AZA as a single agent (P=0.1563) (Figure 4C, upper panel). At the same time, the proportion of healthy CD45.1$^+$ BM cells increased after the treatment with alvocidib + 5-AZA combination, although this effect did not reach significance (P=0.1094) (Figure 4C, lower panel). Histological analyses of BM cells after alvocidib + 5-AZA combination treatment showed a decrease in the percentage of immature blasts and improved development of mature BM cells compared to single 5-AZA treatment (P=0.0391) reflecting the fact that this combination favors eradication of leukemic donor cells in transplanted mice (Figure 4D). In addition, this result also suggested that the combination treatment could have a direct differen-



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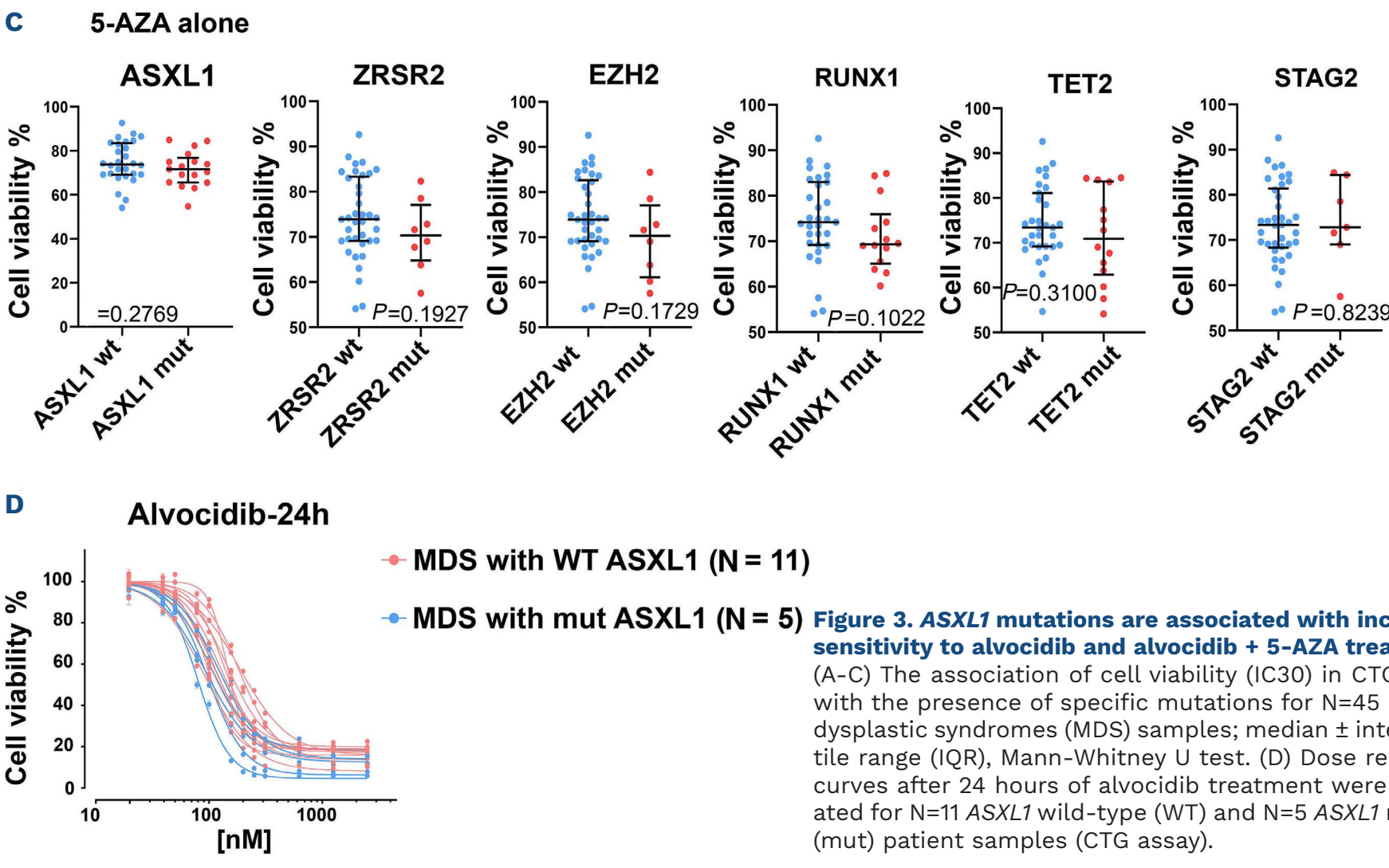


Figure 3. *ASXL1* mutations are associated with increased sensitivity to alvocidib and alvocidib + 5-AZA treatment. (A-C) The association of cell viability (IC30) in CTG assay with the presence of specific mutations for N=45 myelodysplastic syndromes (MDS) samples; median ± interquartile range (IQR), Mann-Whitney U test. (D) Dose response curves after 24 hours of alvocidib treatment were generated for N=11 *ASXL1* wild-type (WT) and N=5 *ASXL1* mutant (mut) patient samples (CTG assay).

Table 2. Multivariable analyses for alvocidib treatment.

Logistic model	Relative risk	95% CI (lower)	95% CI (upper)	*P*
Age ≥70 *vs.* <70 years	1.350	0.208	8.758	0.753
Male *vs.* female	0.476	0.054	4.168	0.503
IPSS-R Very high *vs.* int/high	0.211	0.022	1.998	0.175
ASXL1	0.261	0.041	1.678	0.157
TET2	0.169	0.016	1.813	0.142
RUNX1	0.200	0.022	1.807	0.152
EZH2	0.145	0.002	12.195	0.393
ZRSR2	0.784	0.037	16.648	0.875
STAG2	1.339	0.047	37.833	0.864

CI: confidence interval; IPSS-R: Revised International Prognostic Scoring System; int: intermediate.

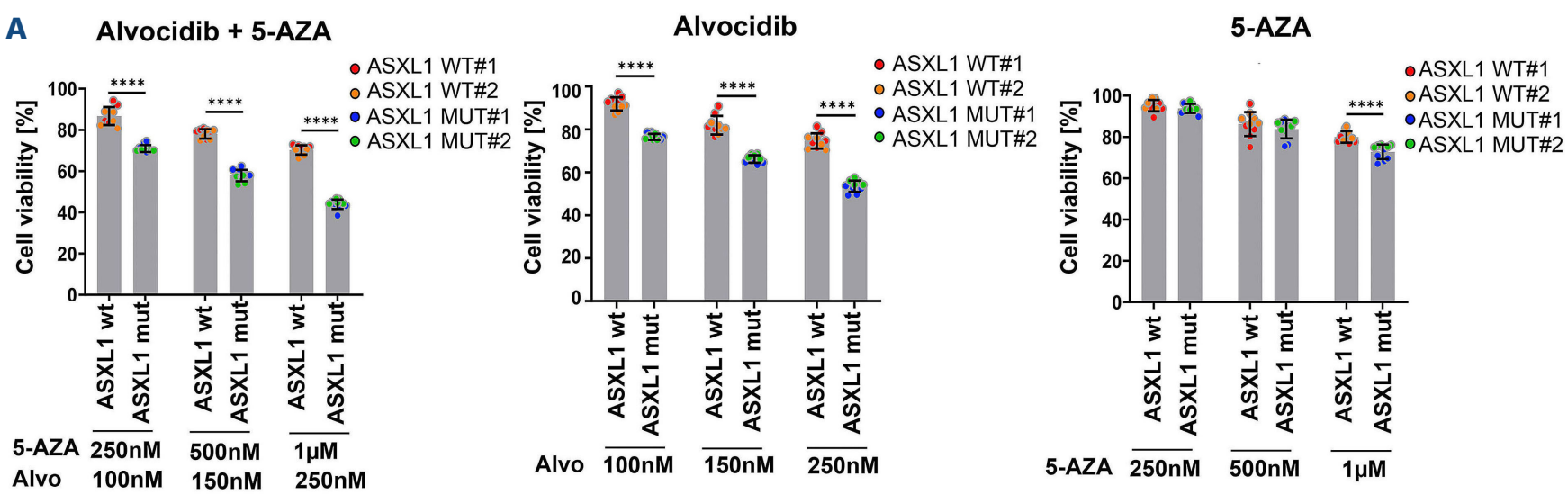
tiating effect on CD34⁺ MDS cells. In order to test this hypothesis, we performed colony-forming unit (CFU) assays after treatment of primary non-expanded CD34⁺ cells from N=5 MDS patients (P_32, P_4, P_7, P_39 and P_35) with single agents or their combination. The patient numbers in CFU assay corresponded to patient numbers provided in Figure 2A-C and *Online Supplementary Table S1*. We determined myeloid and erythroid differentiation of CD34⁺ cells after CFU assay using flow cytometry (*Online Supplementary Figure S11* for gating strategy). Our data showed that in all analyzed patients, CD34⁺ cells were skewed towards myeloid differentiation as assessed by the percentages of CD45⁺CD33⁺ cells (*Online Supplementary Figure S12A*). Only a small proportion of CD45⁻CD235⁺ erythroid cells was present after CFU assay in four of five MDS patients (*Online Supplementary Figure S12B*). Predominant myeloid and inefficient erythroid differentiation of analyzed samples is an expected result since all of these MDS patients had a high-risk disease. However, in patient P_39 (AML-MRC, *ASXL1* WT status) we observed decrease in the percentage of myeloid cells in response to alvocidib

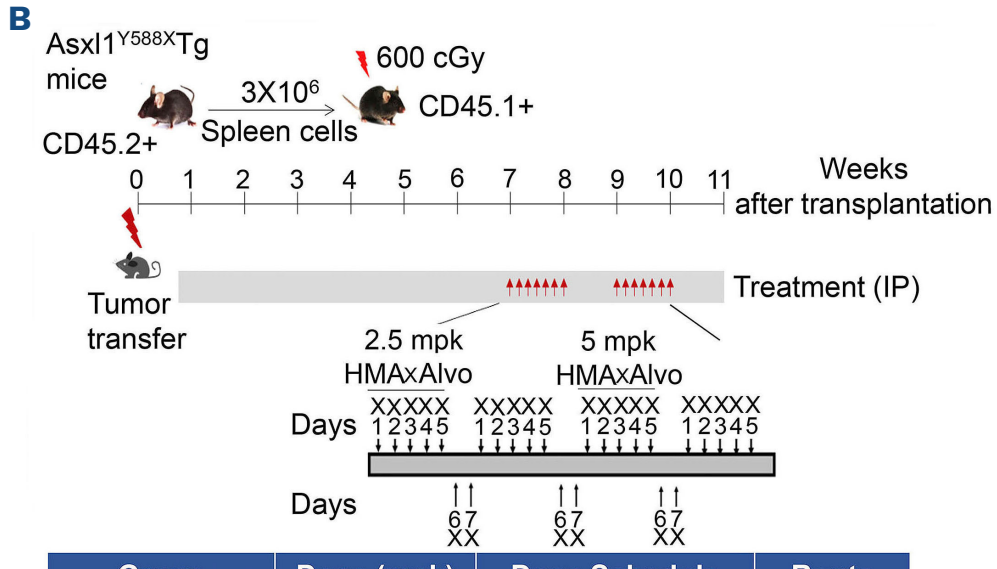
and combination treatment. Concomitantly, this was associated with increase in erythroid differentiation. Significant decrease in myeloid differentiation in response to the alvocidib + 5-AZA treatment was also observed in P_35 (MDS/MPNu, *ASXL1* MUT status). Overall, in addition to cytotoxic effects this result suggests potential differentiating effects of the combination treatment in those patient samples where mutated or residual non-mutated $CD34^+$ cells are still capable of multilineage differentiation.

Table 3. Multivariable analyses for alvocidib + 5-AZA treatment.

Logistic model	Relative risk	95% CI (lower)	95% CI (upper)	*P*
Age ≥70 *vs.* <70 years	2.617	0.352	19.425	0.347
Male *vs.* female	2.857	0.212	38.478	0.429
IPSS-R Very high *vs.* int/high	0.138	0.015	1.239	0.077
ASXL1	0.058	0.004	0.815	0.035
TET2	2.587	0.060	110.653	0.620
RUNX1	0.087	0.005	1.383	0.084
EZH2	1.974	0.007	543.378	0.812
ZRSR2	0.000	0.000	-	0.998
STAG2	6.377	0.110	370.146	0.371

5-AZA: 5-azacytidine; CI: confidence interval; IPSS-R: Revised International Prognostic Scoring System; int: intermediate.





Group	Dose (mpk)	Dose Schedule	Route
5-AZA	1	qdx3	IP
5-AZA + Alvo	1/2.5 or 5	qdx3 / qw (day 5)	IP/IP

Continued on following page.

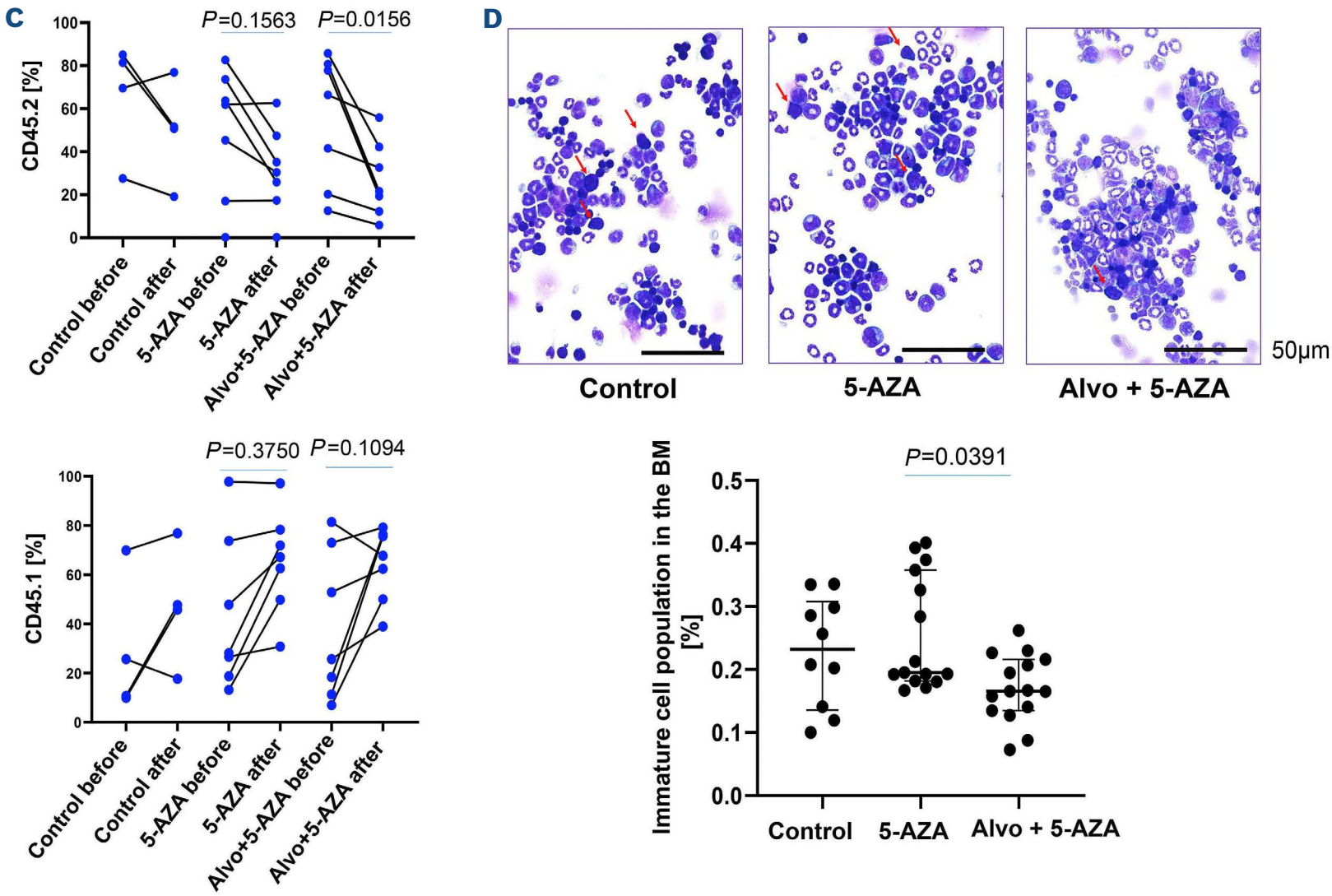


Figure 4. *Asxl1* mutation sensitizes myelodysplastic syndromes cells to alvocidib and alvocidib + 5-AZA treatment in a mouse model. (A) Bone marrow (BM) mononuclear cells (MNC) from N=2 wild-type (WT) and N=2 *Asxl1*^{Y588X} transgenic (Tg) mice were treated with alvocidib (Alvo), 5-azacytidine (5-AZA) or alvocidib + 5-AZA for 8 hours (h). Cell viability was determined using CTG assay; data are combined for N=2 mice and N=5 replicates for each mouse and presented as mean ± standard deviation (SD); unpaired Student's *t* test. (B) Experimental design for *in vivo* assessment of alvocidib + 5-AZA combination in *Asxl1*^{Y588X} Tg mice. (C) The percentages of CD45.1^{+} (healthy recipient mice) and CD45.2^{+} cells (*Asxl1*^{Y588X} Tg leukemic donor mice) in the bone marrow (BM) were analyzed by flow cytometry after treatment with 5-AZA and alvocidib + 5-AZA. The data for individual mice before and after treatment are shown; Wilcoxon matched-pairs signed rank test. (D) Histological analysis of BM cytospins at the treatment endpoint in recipient CD45.1^{+} mice; median ± interquartile range; Kruskal-Wallis test with Dunn's multiple comparisons. Arrows on the histological images indicate immature blasts. IP: intraperitoneal.

ASXL1 mutations are associated with elevated expression of pro-apoptotic factor NOXA

In order to elucidate possible molecular alterations in *ASXL1*-mutated HSPC that sensitize them to alvocidib treatment, we assessed the expression of anti-apoptotic factor MCL-1 and the pro-apoptotic factor NOXA. The interaction of NOXA and MCL-1 was previously reported to result in proteasomal degradation of MCL-1, which sensitizes AML cells to apoptotic stimuli.[21,22] We found that although the expression of the *MCL-1* gene was not altered in *ASXL1*-mutated HSPC, the expression of the *NOXA* gene was significantly increased in *ASXL1-mutated* HSPC as compared to non-mutated HSPC (P=0.0012; Figure 5).

Discussion

HMA, including 5-AZA, are first-line treatments in high-risk MDS patients. Those patients who do not respond or become resistant to HMAs currently have few therapeutic options. In this study, we found that the CDK9 inhibitor alvocidib and 5-AZA exerted additive cytotoxic effects on primary CD34^{+} MDS cells. The cytotoxic responses of MDS samples to the alvocidib and alvocidib + 5-AZA treatment combination were heterogeneous. This heterogeneity in primary CD34^{+} MDS progenitor cells is expected and was shown previously by our own group and others for 5-AZA treatment.[23,24] So far, no robust biomarkers to predict this differential response to 5-AZA have been discovered. Our analysis of the responses to 5-AZA and alvocidib combination revealed that the sensitivity of MDS cells to these drugs was only partially associated with MCL-1 dependency of CD34^{+} HSPC/blasts. Deeper insights into potential biomarkers of response using myeloid panel sequencing of CD34^{+} cells from MDS samples and functional data from transgenic mouse models suggested *ASXL1* mutations to be a candidate biomarker associated with alvocidib + 5-AZA sensitivity. Of note, although *MCL-1* expression in

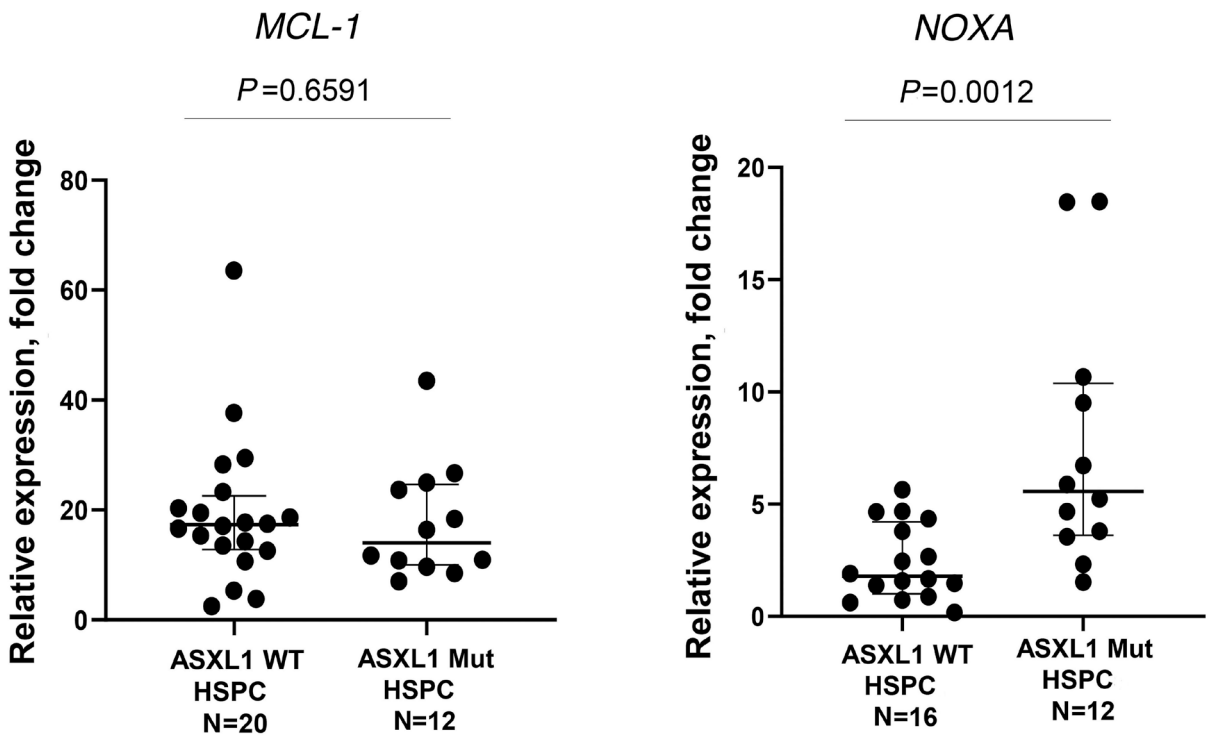


Figure 5. *ASXL1* mutant myelodysplastic syndromes hematopoietic stem and progenitor cells overexpress *NOXA*. The expression of *MCL-1* and *NOXA* genes was assessed in hematopoietic stem and progenitor cells (HSPC) of *ASXL1* wild-type (WT) and *ASXL1*-mutated (mut) patients using real-time quantitative polymerase chain reaction; median ± interquartile range, Mann-Whitney *U* test. Outlier values were removed using GraphPad Prism 8.4.3 software (ROUT method with Q factor =1%).

WT and *ASXL1* samples was comparable, the expression of MCL-1 antagonist *NOXA* was significantly increased in *ASXL1*-mutated samples.

The rationale to perform sequential treatment of MDS samples using 5-AZA followed by alvocidib was based on previous reports demonstrating the ability of 5-AZA to induce the expression of pro-apoptotic MCL-1 interacting protein NOXA after short treatment times of 24-48 h.[5,19,25] Subsequent sequential application of alvocidib for 6-24 h could further inhibit MCL-1 protein expression sensitizing 5-AZA pretreated samples to the combination therapy by skewing the balance toward pro-apoptotic effects of NOXA.[19] However, our *in vitro* experimental data revealed that there were only weak and non-significant correlations between MCL-1 dependency and cytotoxic effects of alvocidib or alvocidib + 5-AZA in higher-risk MDS patient samples. Interestingly, biomarker-driven phase II clinical trials of alvocidib in combination with cytarabine and mitoxantrone in relapsed/refractory AML reported that the composite complete remission (CR) rates were the highest in highly MCL-1 -dependent patients (≥40%) (*clinicaltrials gov. Identifier: NCT02520011*).[26] However, the association of CR with increasing MCL-1 dependency was not observed presumably due to a limited size of the patient cohort.[27] To the best of our knowledge and despite the fact that alvocidib is a non-specific inhibitor of CDK, MCL-1 is the only currently proposed biomarker of alvocidib response. In the light of this data, we aimed to identify additional biomarkers potentially predictive of response to alvocidib or the alvocidib + 5-AZA combination. By correlating mutational profiles of analyzed patient samples with the cell viability in CTG assays, we found *ASXL1* mutations to be an independent predictor of increased drug combination cytotoxic activity in a multivariable analysis. Next, we used a previously generated and characterized *Asxl1*^{Y588X} mouse model to validate the involvement of *Asxl1* mutations in alvocidib and alvocidib + 5-AZA sensitivity.[16] These mice were reported to develop a spectrum of myeloid malignancies, including MDS, MPN and AML. They have shorter survival compared to WT littermates and abnormal blood counts including thrombocytosis, anemia and lymphopenia. Some of the mice show dysplastic features in blood, BM, and spleen cells, including hyposegmented neutrophils with fine nuclear bridging, aberrant nuclear structure, and Howell-Jolly bodies in the erythrocytes. Interestingly, increased activity of alvocidib and alvocidib + 5-AZA combination against *Asxl1*-mutated cells as well as specific elimination of *Asxl1*-mutated cells from the BM by this drug combination was further confirmed in *Asxl1*^{Y588X} mice and CD45.1 recipient mice transplanted with splenic cells of *Asxl1*^{Y588X} mice. It is important to mention that 12-month-old *Asxl1*^{Y588X} mice used for transplantation had AML disease. Therefore, together with our data on the MDS-L mouse model and *in vitro* data on MDS primary samples, the current data suggest that this combination may be effective in both high-risk MDS and AML in preclinical settings. A possible limitation in our murine transplantation experiments is that BM-derived AML donor cells could behave differently as compared to spleen derived AML cells due to differences in the microenvironments of the source tissues. In this study, we only transplanted spleen derived cells but did not compare these with BM-derived cells.

Interestingly, a previous study[16] and our current data show that the activities of both alvocidib and ASXL1 converge on the RNA polymerase II regulation. Mutant ASXL1 protein activates pTEFb (CDK9/cyclin T1) complex that further activates RNA polymerase II, while alvocidib represses RNA polymerase II activity via CDK9 inhibition. In addition, it was previously reported that the loss of ASXL1 triggers an apoptotic response in healthy HSPC.[28] In this study, we observed higher expression of pro-apoptotic NOXA in

human MDS HSPC with *ASXL1* mutations. Since NOXA and NOXA-derived peptides are the most selective antagonists of MCL-1 that decrease its half-life and stability,[29] the overexpression of NOXA may potentiate inhibiting effects of alvocidib on the MCL-1 expression in *ASXL1*-mutated MDS. In this study, we also observed significant associations of ZRSR2 and EZH2 mutations with both alvocidib and alvocidib + 5-AZA sensitivity. However, before elucidating the underlying mechanism, their direct involvement in the drug sensitivity needs to be studied in knock-in/knockout cellular systems or transgenic models for myeloid neoplasms.

It should also be noted that the long-term effects of the treatment with 5-AZA + alvocidib observed *in vivo* in MDS-L xenografted model may also involve reprogramming of BM microenvironment. Indeed, direct modulatory effects of 5-AZA were previously reported on the BM mesenchymal stromal cell compartment.[23] Therefore, potential effects of alvocidib and its combination with HMA on the BM microenvironment should be addressed in the future mechanistic studies.

In summary, our study provides preclinical support for the use of alvocidib in combination with 5-AZA for higher-risk MDS. *ASXL1* mutations should be analyzed as potential stratification markers to select for highly responsive patients in further clinical evaluation.

Disclosures

This study was funded by research support of Sumitomo Pharma Oncology, Inc., Lehi, UT, USA. JMF is an employee of Sumitomo Pharma Oncology, Inc. DTS serves on the scientific advisory board at Kurome Therapeutics; is a consultant for Kymera Therapeutics, Kurome Therapeutics, Captor Therapeutics, and Tolero Therapeutics; has equity in Kurome Therapeutics. AR is a consultant for Taiho Pharmaceuticals and Epizyme. AMA is a consultant for VOR Biopharma and received funding from VOR Biopharma and Actinium Pharmaceuticals. All other authors have no conflicts of interest to disclose.

Contributions

VR and DN designed and conducted the study, analyzed the data and wrote manuscript draft. QX performed experiments and analyzed the data. NS, GG, AW, LB, MW and MD performed experimental studies. AS and J-CJ performed bioinformatics analysis. EA, FR and JMF contributed to the study design. VN, NW, JO, and IP provided technical assistance for sample workup, cell culture, and molecular analyses. AJ and AD provided primary material from healthy controls. CW performed statistical analysis of the data. DTS and F-CY designed and conducted the study. SM, AMA, AR, GM, LS, MJ, and PW provided material from patients and clinical data. DN and W-KH supervised the study and provided research infrastructure.

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Data-sharing statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

1. 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(3):223-232.
2. Silverman LR, Demakos EP, Peterson BL, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol. 2002;20(10):2429-2440.
3. Parker JE, Mufti GJ, Rasool F, et al. The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. Blood. 2000;96(12):3932-3938.
4. Parker JE, Fishlock KL, Mijovic A, et al. 'Low-risk' myelodysplastic syndrome is associated with excessive apoptosis and an increased ratio of pro-versus anti-apoptotic bcl-2-related proteins. Br J Haematol. 1998;103(4):1075-1082.
5. Jin S, Cojocari D, Purkal JJ, et al. 5-Azacitidine induces NOXA to prime AML cells for venetoclax-mediated apoptosis. Clin Cancer Res. 2020;26(13):3371-3383.

6. Mei M, Aldoss I, Marcucci G, Pullarkat V. Hypomethylating agents in combination with venetoclax for acute myeloid leukemia: update on clinical trial data and practical considerations for use. Am J Hematol. 2019;94(3):358-362.
7. Fischer MA, Song Y, Gbyli R, et al. MCL1 dependence across MDS subtypes and dual inhibition of MCL1 and BCL2 in MISTRG6 mice. bioRxiv. 2020:2020.06.05.133090. doi: https://doi.org/10.1101/2020.06.05.133090 [preprint, not peer-reviewed].
8. Chen R, Keating MJ, Gandhi V, Plunkett W. Transcription inhibition by flavopiridol: mechanism of chronic lymphocytic leukemia cell death. Blood. 2005;106(7):2513-2519.
9. Yeh YY, Chen R, Hessler J, et al. Up-regulation of CDK9 kinase activity and Mcl-1 stability contributes to the acquired resistance to cyclin-dependent kinase inhibitors in leukemia. Oncotarget. 2015;6(5):2667-2679.
10. Boffo S, Damato A, Alfano L, Giordano A. CDK9 inhibitors in acute myeloid leukemia. J Exp Clin Cancer Res. 2018;37(1):36.
11. Tibes R, Bogenberger JM. Transcriptional silencing of MCL-1 through cyclin-dependent kinase inhibition in acute myeloid leukemia. Front Oncol. 2019;9:1205.
12. Yin T, Lallena MJ, Kreklau EL, et al. A novel CDK9 inhibitor shows potent antitumor efficacy in preclinical hematologic tumor models. Mol Cancer Ther. 2014;13(6):1442-1456.
13. Lin TS, Heerema NA, Lozanski G, et al. Flavopiridol (alvocidib) induces durable responses in relapsed chronic lymphocytic leukemia (CLL) patients with high-risk cytogenetic abnormalities. Blood. 2008;112(11):46.
14. Zeidner JF, Karp JE. Clinical activity of alvocidib (flavopiridol) in acute myeloid leukemia. Leuk Res. 2015;39(12):1312-1318.
15. Zeidner JF, Lee DJ, Frattini M, et al. Phase I study of alvocidib followed by 7+3 (cytarabine + daunorubicin) in newly diagnosed acute myeloid leukemia. Clin Cancer Res. 2021;27(1):60-69.
16. Yang H, Kurtenbach S, Guo Y, et al. Gain of function of ASXL1 truncating protein in the pathogenesis of myeloid malignancies. Blood. 2018;131(3):328-341.
17. Nakamura S, Ohnishi K, Yoshida H, et al. Retrovirus-mediated gene transfer of granulocyte colony-stimulating factor receptor (G-CSFR) cDNA into MDS cells and induction of their differentiation by G-CSF. Cytokines Cell Mol Ther. 2000;6(2):61-70.
18. Rhyasen GW, Wunderlich M, Tohyama K, et al. An MDS xenograft model utilizing a patient-derived cell line. Leukemia. 2014;28(5):1142-1145.
19. Matsumura Y, Tyagi E, Pathi S, et al. CDK9 inhibition combined with hypomethylating agents target MCL-1 dependency in MDS and AML. Cancer Res. 2021;81(Suppl 13):1959.
20. Foight GW, Ryan JA, Gullá SV, Letai A, Keating AE. Designed BH3 peptides with high affinity and specificity for targeting Mcl-1 in cells. ACS Chem Biol. 2014;9(9):1962-1968.
21. Arai S, Varkaris A, Nouri M, et al. MARCH5 mediates NOXA-dependent MCL1 degradation driven by kinase inhibitors and integrated stress response activation. Elife. 2020;9:e54954.
22. Chiou JT, Huang NC, Huang CH, et al. NOXA-mediated degradation of MCL1 and BCL2L1 causes apoptosis of daunorubicin-treated human acute myeloid leukemia cells. J Cell Physiol 2021;236(11):7356-7375.
23. Wenk C, Garz AK, Grath S, et al. Direct modulation of the bone marrow mesenchymal stromal cell compartment by azacitidine enhances healthy hematopoiesis. Blood Adv. 2018;2(23):3447-3461.
24. Xu Q, Streuer A, Jann JC, et al. Inhibition of lysyl oxidases synergizes with 5-azacytidine to restore erythropoiesis in myelodysplastic and myeloid malignancies. Nat Commun. 2023;14(1):1497.
25. Cojocari D, Smith BN, Purkal JJ, et al. Pevonedistat and azacitidine upregulate NOXA (PMAIP1) to increase sensitivity to venetoclax in preclinical models of acute myeloid leukemia. Haematologica. 2022;107(4):825-835.
26. Zeidner JF, Lin TL, Vigil CE, et al. A prospective biomarker analysis of alvocidib followed by cytarabine and mitoxantrone in MCL-1-dependent relapsed/refractory acute myeloid leukemia. Blood Cancer J. 2021;11(10):175.
27. Zeidner JF, Lee DJ, Fine G, et al. Zella 201: a biomarker-guided phase II study of alvocidib followed by cytarabine and mitoxantrone in MCL-1 dependent acute myeloid leukemia (AML): results of newly diagnosed high-risk exploratory arm. Blood. 2020;136(Suppl 1):48-50.
28. Hilgendorf S, Folkerts H, Schuringa JJ, Vellenga E. Loss of ASXL1 triggers an apoptotic response in human hematopoietic stem and progenitor cells. Exp Hematol. 2016;44(12):1188-1196.
29. Guikema JE, Amiot M, Eldering E. Exploiting the pro-apoptotic function of NOXA as a therapeutic modality in cancer. Expert Opin Ther Targets. 2017;21(8):767-779.