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ORIGINAL ARTICLE

HOX gene expression predicts response to BCL-2 inhibition in acute myeloid leukemia

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Inhibitors of B-cell lymphoma-2 (BCL-2) such as venetoclax (ABT-199) and navitoclax (ABT-263) are clinically explored in several cancer types, including acute myeloid leukemia (AML), to selectively induce apoptosis in cancer cells. To identify robust biomarkers for BCL-2 inhibitor sensitivity, we evaluated the *ex vivo* sensitivity of fresh leukemic cells from 73 diagnosed and relapsed/refractory AML patients, and then comprehensively assessed whether the responses correlated to specific mutations or gene expression signatures. Compared with samples from healthy donor controls (nonsensitive) and chronic lymphocytic leukemia (CLL) patients (highly sensitive), AML samples exhibited variable responses to BCL-2 inhibition. Strongest CLL-like responses were observed in 15% of the AML patient samples, whereas 32% were resistant, and the remaining exhibited intermediate responses to venetoclax. BCL-2 inhibitor sensitivity was associated with genetic aberrations in chromatin modifiers, *WT1* and *IDH1/IDH2*. A striking selective overexpression of specific *HOXA* and *HOXB* gene transcripts were detected in highly BCL-2 inhibitor sensitive samples. *Ex vivo* responses to venetoclax showed significant inverse correlation to β2-microglobulin expression and to a lesser degree to *BCL-XL* and *BAX* expression. As new therapy options for AML are urgently needed, the specific *HOX* gene expression pattern can potentially be used as a biomarker to identify venetoclax-sensitive AML patients for clinical trials.

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INTRODUCTION

Although the prognosis of acute myeloid leukemia (AML) has improved over the past decades, chemotherapy is curative only in 35 to 40% of adult patients who are ⩽60 years of age and in 5 to 15% of patients who are >60 years of age.[1] Although new therapy options hold promise to improve treatment outcomes, a major challenge will be to identify predictive biomarkers for response, allowing use of targeted agents in patients most likely to benefit, and also enabling the design of new combinatorial therapies.[2]

In lymphatic diseases the inhibition of anti-apoptotic B-cell lymphoma-2 (BCL-2) proteins has been widely explored with promising results.[3–5] BCL-2 family proteins play a critical role in the regulation of apoptosis by regulating both cell survival and apoptosis. BCL-2 and BCL-XL promote survival by blocking BH3-selective activators (BIM, BID, BAD and PUMA) and their multidomain targets (BAX and BAK), thus preventing mitochondrial outer membrane apoptotic pore formation.[6,7] BH3 mimetic drugs resemble the shared BH3 domains of sensitizer proteins and prevent their binding to anti-apoptotic proteins.[8,9] In clinical trials, inhibition of BCL-XL by navitoclax (ABT-263) has resulted in severe on-target thrombocytopenia, hampering clinical development.[10] This has led to the development of the second-generation BH3 mimetic venetoclax (ABT-199), a compound that has 5-fold higher binding affinity for BCL-2 and >800-fold lower affinity for BCL-XL, and thus exhibits minimal effects on thrombopoiesis.[9]

In AML, previous studies have shown that the expression and protein levels of anti-apoptotic proteins BCL-2, BCL-XL and MCL-1 (myeloid cell leukemia 1) are highly variable, reflecting to some extent disease prognosis.[11–13] Previously, BCL-2 inhibitors have been explored in AML cell lines and primary patient cells and protein levels of BCL-2, BCL-XL and MCL-1 have been correlated to venetoclax sensitivity.[14] Recently, mutations in *IDH1* or *IDH2* were shown to induce venetoclax sensitivity by (R)-2-hydroxyglutarate-mediated inhibition of cytochrome *c* oxidase (COX) activity in the mitochondrial electron transport chain.[15] COX inhibition led to a lower mitochondrial threshold, thus sensitizing blasts to venetoclax. Comprehensive data predicting sensitivity to BCL-2 inhibition in AML are limited and robust biomarkers are needed to select patients most likely to benefit from therapy.

In this study, we explored BCL-2 inhibitor sensitivity *ex vivo* in a cohort of 28 newly diagnosed and 45 relapsed/refractory fresh AML patient samples with extensive molecular and functional profiling data to discover putative biomarkers for predicting sensitivity. As venetoclax resistance is associated with elevated BCL-XL expression levels, we also wanted to explore whether dual inhibition of BCL-2 and BCL-XL by navitoclax generates deeper responses than the more selective BCL-2-only inhibitor venetoclax. *Ex vivo* cancer-selective responses were identified by computing selective drug sensitivity scores (sDSS)[16] using fresh leukemic blasts from AML patients. Whole-exome and transcriptome sequencing and targeted real-time quantitative reverse transcriptase

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PCR (RQ-PCR) were used for biomarker discovery. We detected responses in both diagnostic and relapsed/refractory samples and found mutations in chromatin modifier genes, *WT1* and *IDH1* and *IDH2* to predict sensitivity. Importantly, we observed that a specific *HOX* gene expression profile predicted venetoclax sensitivity, and absent or low *HOX* gene expression predicted resistance.

MATERIALS AND METHODS

Patient material

A total of 73 bone marrow (BM) aspirates and peripheral blood samples (leukemic cells) and skin biopsies (nonmalignant cells for germline genomic information) from 57 AML patients were collected after signed informed consent from each patient (permit numbers 239/13/03/00/2010, 303/13/03/01/2011, Helsinki University Hospital Ethics Committee) in accordance with the Declaration of Helsinki. In addition, BM aspirates from different healthy donors (12 for navitoclax testing and 7 for venetoclax), and 3 CLL patients were obtained. Patient characteristics are summarized in Supplementary Table 1. Mononuclear cells (MNCs) were isolated by Ficoll density gradient separation (GE Healthcare, Little Chalfont, UK), washed, counted and suspended in Mononuclear Cell Medium (PromoCell, Heidelberg, Germany) supplemented with 0.5 µg/ml gentamicin. One sample from patient 393, a secondary AML after myelodysplastic syndrome (MDS) with 20% myeloblasts, was enriched for the $CD34^+$ cell population (sample 393_3, corresponding to the blast cell population) using paramagnetic beads according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany).

Drug sensitivity and resistance testing

The *ex vivo* drug sensitivity of AML BM or peripheral blood blast cells was assessed against venetoclax ($n = 47$) and navitoclax ($n = 72$) as previously described.[17] In short, the drugs were preplated in 384-well plates over a 10 000-fold-concentration range (1–10 000 nM for both venetoclax and navitoclax in 5 concentrations) with 10 000 cells added to each well. After a 3-day incubation at 37 °C, cell viability was measured using the CellTiter-Glo reagent (Promega, Madison, WI, USA). Dose response curves for each drug were generated for the patient cells, whereas BM MNC fractions from healthy donors served as controls. DSS and sDSS were calculated as previously described.[16,17] Briefly, DSS is a measure of drug response based on the area under the dose response curve that captures both the potency and the efficacy of the drug effect. It integrates complementary information extracted by half-maximal inhibitory concentration (IC_{50}), slope and minimal and maximum asymptotes. sDSS reflects the difference in leukemia cell response compared with the median response in healthy donor BM MNCs (leukemia-selective response).

Exome sequencing and somatic mutation analysis

Genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Exome capture was performed using the Nimblegen SeqCap EZ v2 (Roche NimbleGen, Madison, WI, USA), Agilent SureSelect v5 Exome or Agilent SureSelect XT Clinical Research Exome (Agilent, Santa Clara, CA, USA) capture kits and the HiSeq 1500 or 2500 instruments (Illumina, San Diego, CA, USA).

Exome sequence reads were processed and aligned to the GRCh37 human reference-genome primary assembly as previously described.[18] Somatic-mutation calling was done for the exome-capture target regions and the flanking 500 bp. High confidence somatic mutations were called for each tumor sample using the VarScan2 somatic algorithm[19] with the following parameters: strand filter 1, min coverage normal 8, min coverage tumor 6, somatic *P*-value 1, normal purity 1 and min var freq 0.05. Mutations were annotated with SnpEff 4.0 (Cingolani *et al.*[20]) using the Ensembl v68 annotation database (European Bioinformatics Institute, Hinxton, UK). To filter out misclassified germline variants, common population variants included in dbSNP database version 130 (National Center for Biotechnology Information, Bethesda, MD, USA) were removed. The remaining mutations were visually validated using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA, USA).

Library preparation, sequencing and data analysis of transcriptomes

For gene expression analysis, total RNA (2.5 to 5 µg) isolated from the AML patient MNCs was depleted of ribosomal RNA (Ribo-Zero rRNA Removal Kit, Epicentre, Madison, WI, USA) and remaining RNA reverse transcribed to complementary DNA (cDNA; SuperScript Double-Stranded cDNA Synthesis Kit, Life Technologies, Carlsbad, CA, USA). RNA-sequencing libraries were prepared by Illumina-compatible Nextera Technology (Epicentre, Madison, WI, USA) and sequenced on the Illumina HiSeq 1500 or 2500 instruments. Sequenced reads were filtered and aligned to the GRCh37 human reference-genome using TopHat. Mapped reads were counted for each genomic feature (gene) with the FeatureCount read-summarization program from the Subread package (WEHI, Melbourne, Australia).[21] The trimmed mean of M-value method from the edgeR package was applied to normalize the raw read count and to determine differential gene expression signatures between sensitive and resistant samples from 53 893 (Ensembl 67) genes.[22]

RQ-PCR

Total RNA was prepared from BM or peripheral blood MNCs using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared from total RNA using SuperScript III reverse transcriptase and random primers (Life Technologies) in a 20 µl reaction, including 40 U RiboLock RNase inhibitor (Thermo Scientific, Waltham, MA, USA). Reference genes, *GAPDH* and *PGK1*, were chosen based on uniform expression in all samples. RQ-PCR was performed for *B2M*, *BCL-2*, *BCL-XL*, *MCL-1*, *BIK*, *BAX*, *BAK1*, *BID*, *BCL2L12*, *BCL2L11* (*BIM*), *BCL2A1*, *BBC3* (*PUMA*) and *BAD* as well as *HOXA1-A7*, *HOXA9*, *HOXA10–A13*, *HOXB1-B9* and *HOXB13* mRNAs. Primer sequences are listed in Supplementary Table 2. RQ-PCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), and the specificities of the amplification products verified by melting curve analysis. Gene expression was quantified using the Pfaffl method based on calculated primer efficiencies on BCL2 family genes and the $\Delta\Delta Cq$ method for the analysis of HOX genes.[23]

Statistical analysis

Statistical analyses were performed with Prism software version 6.0 (GraphPad Software, San Diego, CA, USA). Data sets were subjected to normality testing using the Shapiro–Wilk normality test. Differences between responses modeled by Gaussian distribution were analyzed by *t*-test; otherwise, Mann–Whitney *U* or Wilcoxon matched-pairs signed rank test was used. Correspondingly, statistical dependence between two variables was assessed by Pearson's correlation or Spearman's rank correlation coefficient modeling. All tests were two tailed and *P*-values of < 0.05 were considered statistically significant.

RESULTS

BCL-2 inhibitors are more effective against AML compared with healthy controls cells

We first explored the effect of BCL-2 inhibition in fresh, healthy BM MNCs and detected low sensitivity. The median half-maximal effective concentration (EC_{50}) for venetoclax was 355 nM (7 healthy controls) and for navitoclax 97 nM (12 healthy controls). We used DSS to compare responses between healthy donors and AML cells to venetoclax and navitoclax. DSS is a measure of drug response, a modified area under the curve calculation that integrates all four curve fitting parameters, thereby capturing both the potency and efficacy of the drug. sDSS reflects the difference in observed response compared with healthy donors (leukemia-selective response).[16,17] Higher values represent greater sensitivity. A complete list of EC_{50}, DSS and sDSS values for all samples are presented in Supplementary Table 3. Both venetoclax and navitoclax showed AML-selective responses, with the BCL-2-selective venetoclax having a slightly weaker effect in control MNCs than the dual BCL-2/BCL-XL inhibitor navitoclax (median DSS 7.3 vs 10.2, paired *t*-test $P = 0.03$; Figures 1a and b).

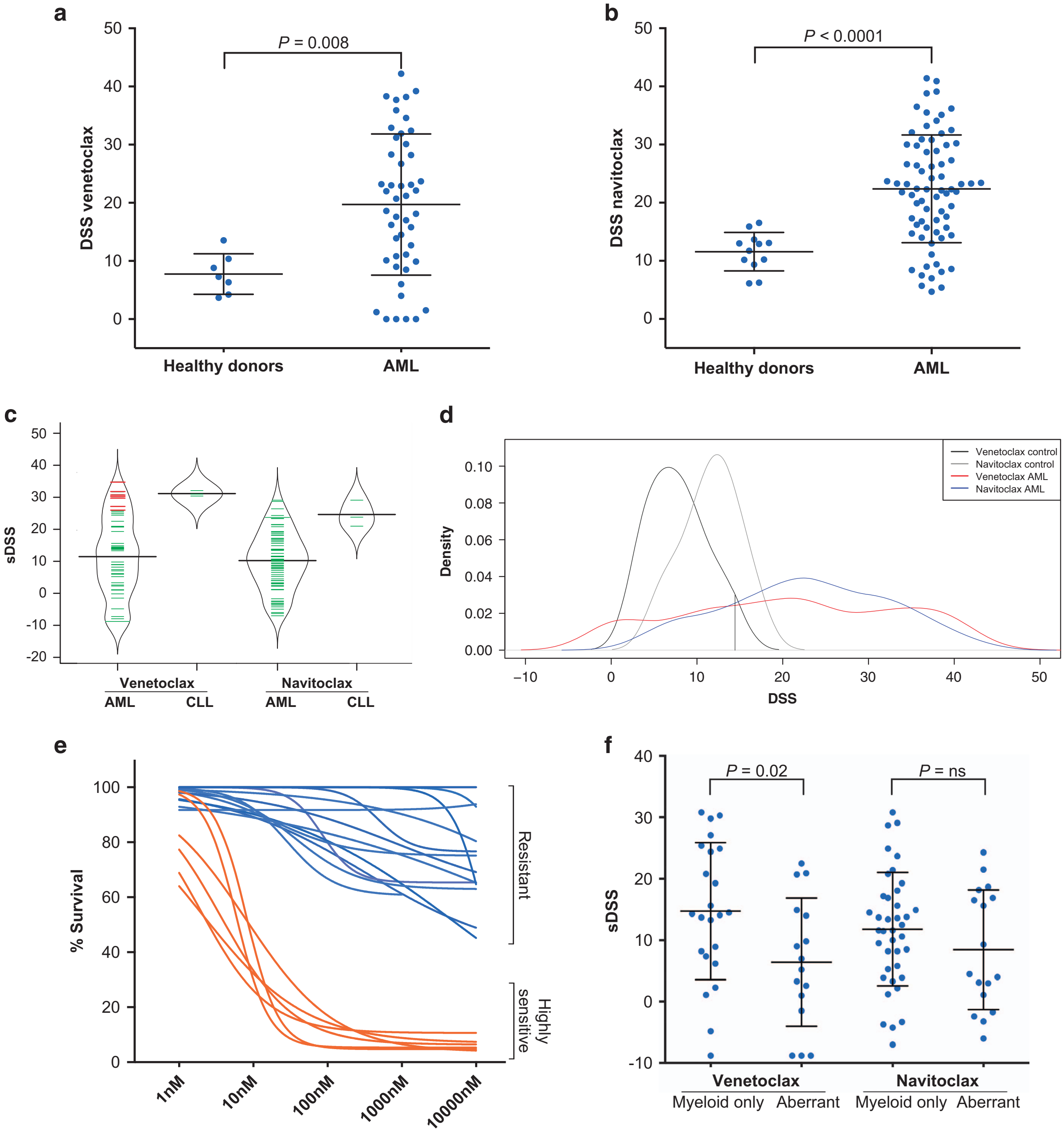

Figure 1. The responses of venetoclax and navitoclax in healthy controls, CLL and AML. Both (**a**) venetoclax and (**b**) navitoclax exhibit AML-selective responses. Mean, s.d. and results of unpaired *t*-test are shown. (**c**) Responses of AML and CLL primary cells to venetoclax and navitoclax. Strongest CLL-like responses were observed in 15% of venetoclax-tested samples (red bars). (**d**) Distribution of venetoclax and navitoclax responses in healthy controls and AML samples. Resistant groups were distinguished by the upper confidence level (95%) of median DSS in healthy controls. Upper confidence level (95%; DSS 13.5) of venetoclax is shown. (**e**) Venetoclax dose response curves of highly sensitive samples and resistant samples. (**f**) Venetoclax and navitoclax sensitivity in samples expressing myeloid-only antigens and in samples also expressing aberrant lymphatic antigens (CD2, CD7, CD10, CD19, CD22, nTdT, CD56, CD79a) on myeloid blasts. Mean for venetoclax: myeloid-only 14.7, aberrant 6.4; for navitoclax: 11.3 vs 8.4, respectively. Results of unpaired *t*-test shown. Immunophenotypic data were obtained from clinical laboratories.

AML responses to venetoclax can be divided into three subgroups

The median EC_{50} value for venetoclax across our cohort in 47 AML patient samples was 31 nM, whereas for navitoclax the median EC_{50} in 72 AML patient samples was 55 nM (Supplementary Table 3). The responses from three fresh primary CLL patient samples were used as a positive control.[9] The median EC_{50} values for the CLL samples were in the low nM range (venetoclax: EC_{50} 1.3, 1.0 and 10.1 nM, respective sDSS values 31.0, 30.4 and 32.1; navitoclax: EC_{50}: 5.7, 7.1 and 16.6 nM and respective sDSS 23.8, 21.0 and 29.1) (Figure 1c).

Compared with the healthy donor and CLL cohorts, primary, fresh AML samples exhibited wide-ranging responses to BCL-2 inhibition, and could be divided into three subgroups based on sensitivity to venetoclax. Resistant AML samples exhibited lower or comparable responses with those observed in healthy donors and thus lacked a leukemia-selective response. The resistant group was distinguished by the upper confidence level (95%) of median DSS for venetoclax (DSS 13.5 translating to sDSS 5.8) in healthy controls (Figure 1d). The highly sensitive subgroup was defined by BCL-2 inhibitor sensitivity similar to that observed in CLL patient samples (Figure 1c). This grouping determined distinct response profiles in 7/47 (15%) highly sensitive and 15/47 (32%) resistant samples (Figure 1e). The intermediate sensitive group exhibited sensitivity in between the resistant and highly sensitive samples (sDSS 5.8–25.5). The grouping was used to help determine whether specific biomarkers correlated with the responses based on molecular profiling analyses.

No correlation was seen between any response group and clinical parameters associated with high proliferation rate (peripheral blood leukocyte count, lactate dehydrogenase) or BM blast count (Supplementary Figure 1). Moreover, we did not observe elevated expression of aberrant lymphoid antigens on blast cells in sensitive samples. Instead, the samples expressing aberrant lymphatic antigens tended to exhibit lower responsiveness to venetoclax (Figure 1f).

AML blasts exhibit similar responses to both venetoclax and navitoclax

We next evaluated whether dual inhibition of BCL-2 and BCL-XL by navitoclax would generate different responses than the more selective BCL-2-only inhibitor venetoclax. The responses between navitoclax and venetoclax were highly correlated (Pearson's r 0.88, $P < 0.0001$), and there were no differences in responses observed in all AML patient samples between the two drugs (Figures 2a and b). Diagnostic samples showed marginally higher responsiveness to venetoclax, whereas refractory samples were slightly more responsive to navitoclax (Figure 2c).

The majority of diagnostic (13/19, 68%) as well as relapsed/refractory (19/28, 67%) samples exhibited intermediate or high sensitivity to venetoclax (Figure 2d). To examine the effect of preceding hematological disease, we evaluated responses in both *de novo* and secondary leukemias with antecedent hematological disease. Although not statistically significant, the diagnostic samples showed higher venetoclax sensitivity compared with samples with secondary etiology (median for venetoclax sDSS in *de novo* AML samples 17.5 and secondary 6.1; Figure 2e). Including only those samples with antecedent MDS or chronic myelomonosytic leukemia (CMML) to the analysis, the result remained similar, presenting a trend toward lower responsiveness in post-MDS/CMML AML samples (median for venetoclax sDSS in *de novo* AML samples 13.7 and secondary AML from MDS/CMML 3.2, P = nonsignificant). In navitoclax-tested samples the difference was statistically significant (median for navitoclax sDSS in *de novo* AML samples 15.9 and secondary AML from MDS/CMML 5.3, $P = 0.04$).

Specific *HOX* gene expression profile predicts venetoclax sensitivity

RNA-sequencing data from three highly sensitive ('CLL-like' responses) and four resistant samples were analyzed to identify possible biomarkers for venetoclax sensitivity in an unbiased manner. Multidimensional scaling plots were generated to visualize the differences between the expression profiles of different samples in two dimensions. The distances between samples corresponding to leading biological coefficient of variation are shown in Supplementary Figure 2A. The sensitive samples showed more homogenous expression profiles than the resistant samples. Normalized read counts were used to determine differential gene expression between venetoclax highly sensitive and resistant groups. The analysis resulted in 322 differentially expressed genes between sensitive and resistant samples with a false discovery rate of < 0.05 (Supplementary Figure 2B). Next, 41 and 281 overexpressed genes in the sensitive and resistant groups, respectively, were further analyzed for their biological function and class. This analysis showed that several *HOX* family genes had significantly higher expression in venetoclax-sensitive samples as compared with venetoclax-resistant samples (Supplementary Figure 2C). Interestingly, gene expression analysis of 16 samples with RNA-sequencing data revealed a general overexpression of *HOXA* and *HOXB* genes in highly sensitive samples and lack or low expression in resistant samples. The samples exhibiting intermediate responses to venetoclax mainly clustered between these two groups (Figure 3a).

To further explore the genes with positive or negative correlation with the drug responses and to validate results from RNA-sequencing, we performed RQ-PCR on 35 samples with available cDNA. As a control, we used sample 1064_3 that showed resistance to venetoclax. Corresponding to the RNA-sequencing results, by RQ-PCR we detected positive correlation of venetoclax response and *HOX* expression in several *HOX* family genes: *HOXA3*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9*, *HOXA11*, *HOXB2*, *HOXB4*, *HOXB4*, *HOXB5* and *HOXB6* (Supplementary Table 4). To differentiate biological variances better, we further compared resistant with highly sensitive samples. We observed significant correlation of seven *HOX* genes (*HOXA2*, *HOXA3*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9* and *HOXB2)* with venetoclax sensitivity (Figure 3b).

Responses to BCL-2 inhibition correlate to mutations in chromatin modifiers, *IDH1/2* and *WT1*

To investigate the possible association of BCL-2 inhibitor response to somatic, nonsynonymous mutations in individual genes or sets of genes characterized by functional similarities as presented previously by The Cancer Genome Atlas Research Network,[24] we explored exome sequencing data from 48 samples (Figure 4). All six samples with *IDH1* or *IDH2* mutations displayed sensitivity to BCL-2 inhibitors. Five of the responsive samples clustered among the intermediate responsive group, whereas one sample clustered in the highly sensitive group, indeed being the most sensitive sample of all (other somatic mutations in this sample were *FLT3-ITD* and *NPM1*). Interestingly, 7/8 samples with an aberration in a chromatin modifier (3/3 samples with *MLL* fusions, 3/3 samples with *NUP98–NSD1* translocation and 1 sample with *ASXL1* mutation) exhibited sensitivity to BCL-2 inhibitors. The only sample non-responsive to BCL2 inhibition (1064_5) had a *KAT6B* mutation.

Responses to navitoclax and venetoclax were detected across all mutational risk groups (that is, low, intermediate and high), and the magnitude of response did not correlate to any specific risk group. Out of 73 samples, 15 (21%) had complex karyotype either at the time of sampling (8 samples) or in previous G-stain examination (7 samples) (Supplementary Table 1). We further evaluated possible differences in venetoclax responses in complex and noncomplex karyotypes. The median sDSS for complex karyotype samples was 8.9 and for non-complex karyotype it was 13.7. The difference was not statistically significant ($P = 0.23$) (Supplementary Figure 3). The responses were also observed in complex karyotype samples with monosomal karyotype. In fact, 4/6 samples with loss of function of *TP53* and/or *17p* deletion showed sensitivity to BCL-2 inhibition. One patient (3443; samples 3443_3 and 3443_6) with observed heterozygous *17p* deletion was refractory to BCL-2 inhibition.

Expression of β2-microglobulin inversely correlates to venetoclax sensitivity

As BCL-2, BCL-XL and MCL-1 protein levels have previously been correlated to venetoclax sensitivity in AML and MDS cells,[14,25] and BCL-2 family gene expression did not show strong correlation to venetoclax sensitivity, we validated the expression of 12 BCL-2 family genes, both pro- and anti-apoptotic (*BCL-2*, *BCL-XL*, *MCL-1*, *BIK*, *BAX*, *BAK1*, *BID*, *BCL2L12*, *BCL2L11* (*BIM*), *BCL2A1*, *BBC3* (*PUMA)* and *BAD*) along with β2-microglobulin (*B2M*) and controls (*GAPDH* and *PGK1*), using RNA from 40 samples. In our experiments, *BCL-XL* RNA levels inversely correlated with venetoclax sensitivity as expected and low expression was observed in highly sensitive samples (Figures 5a and b). However, *BCL-2* or *MCL-1* RNA levels did not correlate to sensitivity (Supplementary Table 4). A positive

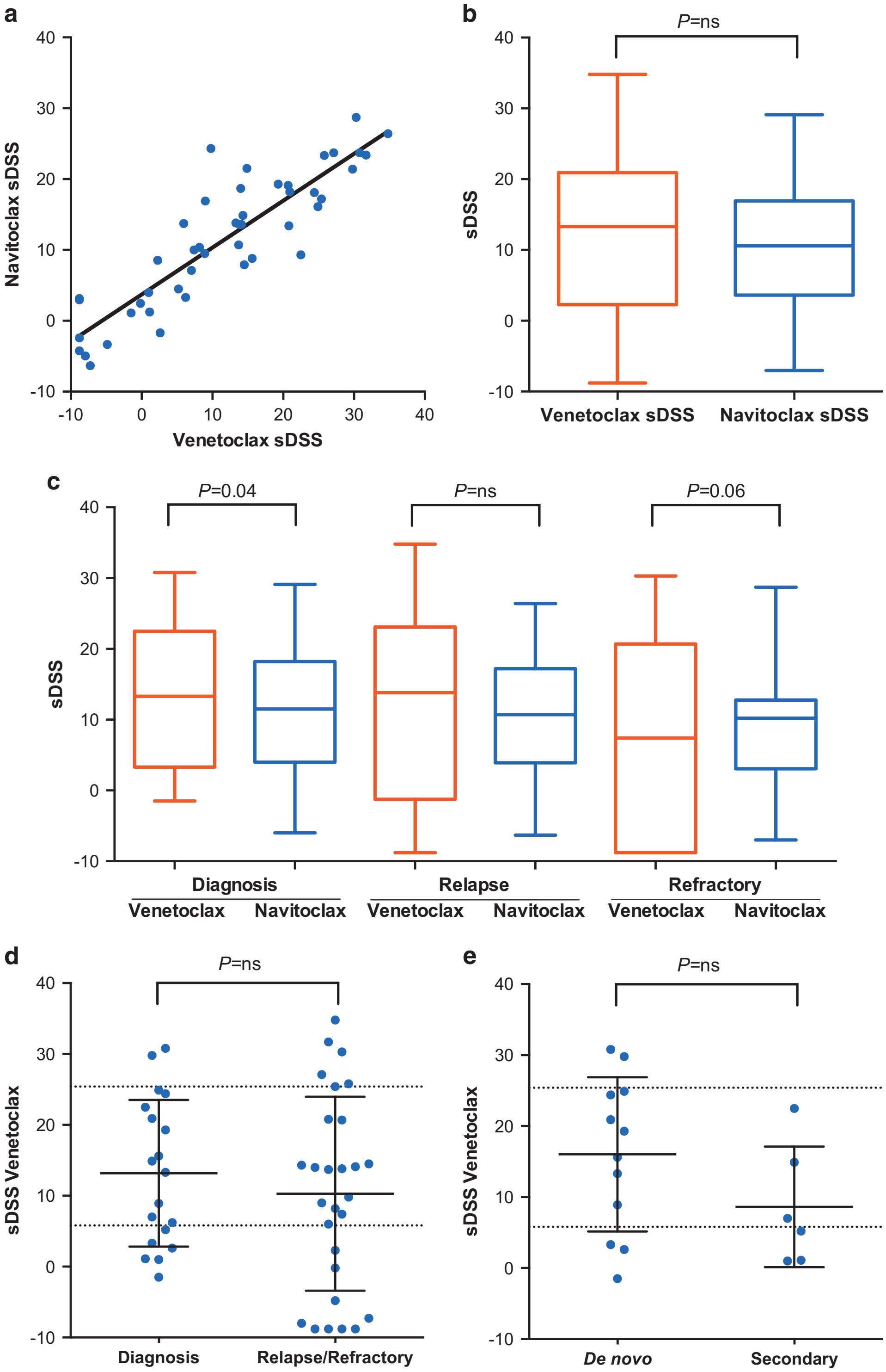

Figure 2. The correlation of BCL2 inhibitor responses in AML and response to venetoclax in different disease stages. (**a**) The correlation of navitoclax and venetoclax responses in AML samples. Pearson's r 0.88, $P < 0.0001$. (**b**) Paired responses observed in all samples were highly similar with no statistical difference. (**c**) In diagnostic samples venetoclax exhibited marginally higher responsiveness (mean 13.2 vs 11.3), whereas in refractory samples navitoclax showed slightly better responses (mean 9.1 vs 7.9). Results of paired *t*-test are shown. (**d**) The majority of diagnostic (13/19, 68%) as well as relapsed/refractory (19/28, 67%) samples exhibited responses to venetoclax. (**e**) The effect of preceding hematological disease on venetoclax sensitivity. In diagnostic samples with secondary etiology, the median for venetoclax was sDSS 6.1 and in *de novo* AML samples it was 17.5. Mann–Whitney *U*-test $P = 0.17$. In (**d**) and (**e**) the means, s.d. and thresholds for intermediate (sDSS 5.8) and highly sensitive (sDSS 25.5) responses are shown.

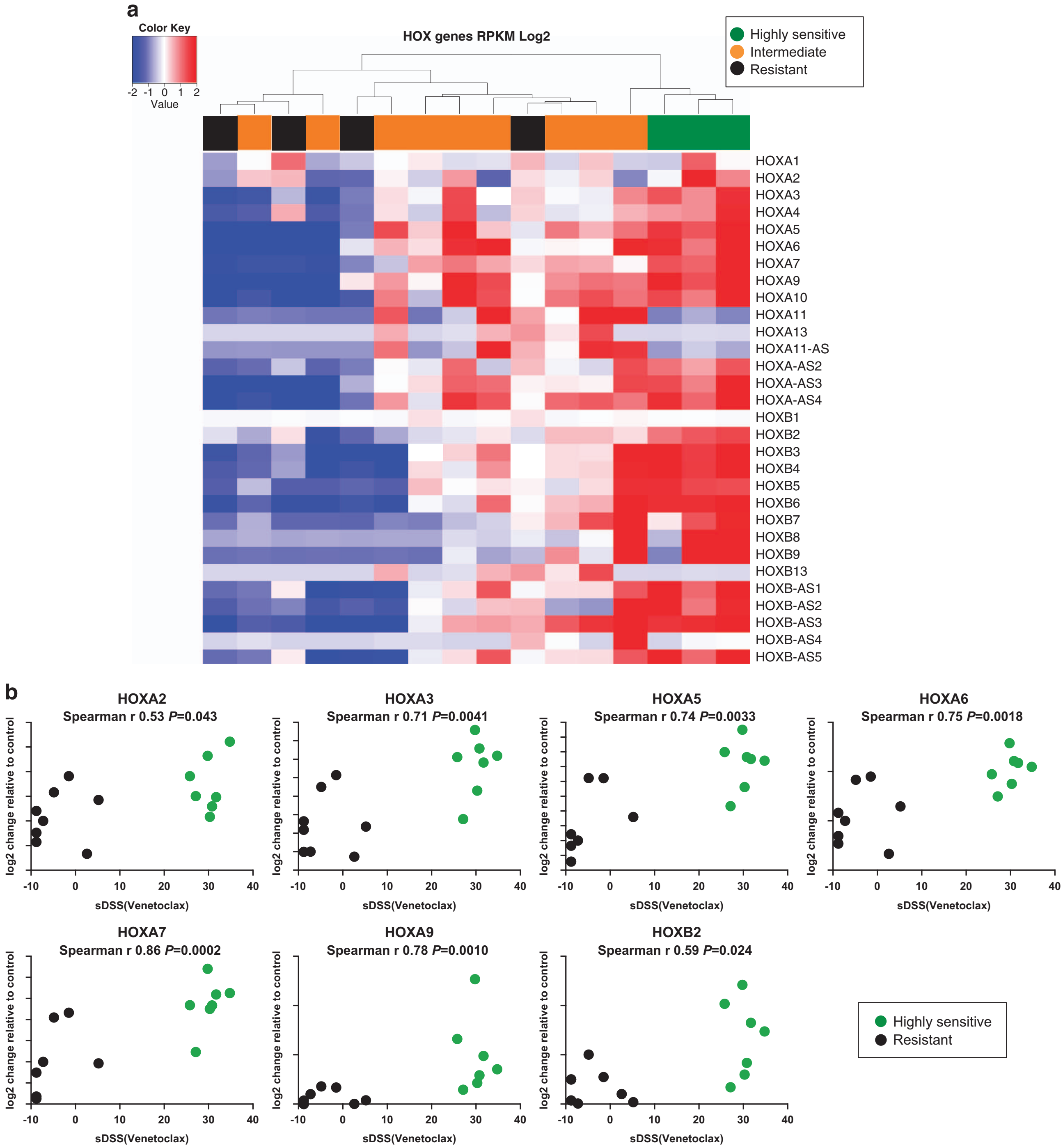

Figure 3. Highly sensitive samples are characterized by a specific *HOX* gene expression profile. (**a**) Expression of *HOXA* and *HOXB* family genes in 16 AML samples (3 highly sensitive, 9 intermediate, 4 resistant samples) derived from RNA-sequence data. Mean centered log2 RPKM (Reads Per Kilobase of transcript per Million mapped reads) were used to cluster samples using Euclidean distance measures. (**b**) The correlation of expression levels of *HOXA2*, *HOXA3*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9* and *HOXB2* determined by RQ-PCR with venetoclax sensitivity in resistant (black) and highly sensitive (green) samples. Results of Spearman's rank correlation coefficient and *P*-values are presented.

correlation occurred between expression of the pro-apoptotic gene *BAX* and venetoclax sensitivity (Figures 5c and d). Inverse correlation of pro-apoptotic *PUMA* (Figures 5e and f) and *BIM* (Supplementary Table 4) expression to venetoclax sensitivity was also observed. Among other tested genes, the strongest correlation was seen between venetoclax response and *B2M* expression where resistant samples exhibited significantly higher *B2M* expression than the highly responsive group (Figures 5g and h).

DISCUSSION

In this study we performed comprehensive genomic and transcriptomic analyses to evaluate factors predicting BCL-2 inhibitor sensitivity in AML. AML patient sample sensitivity was assessed against venetoclax in 47 samples and navitoclax in 72 samples. Only fresh samples were used as extensive processing (for example, cryopreserving and thawing) may cause mitochondrial loading of pro-apoptotic proteins, thus possibly amplifying

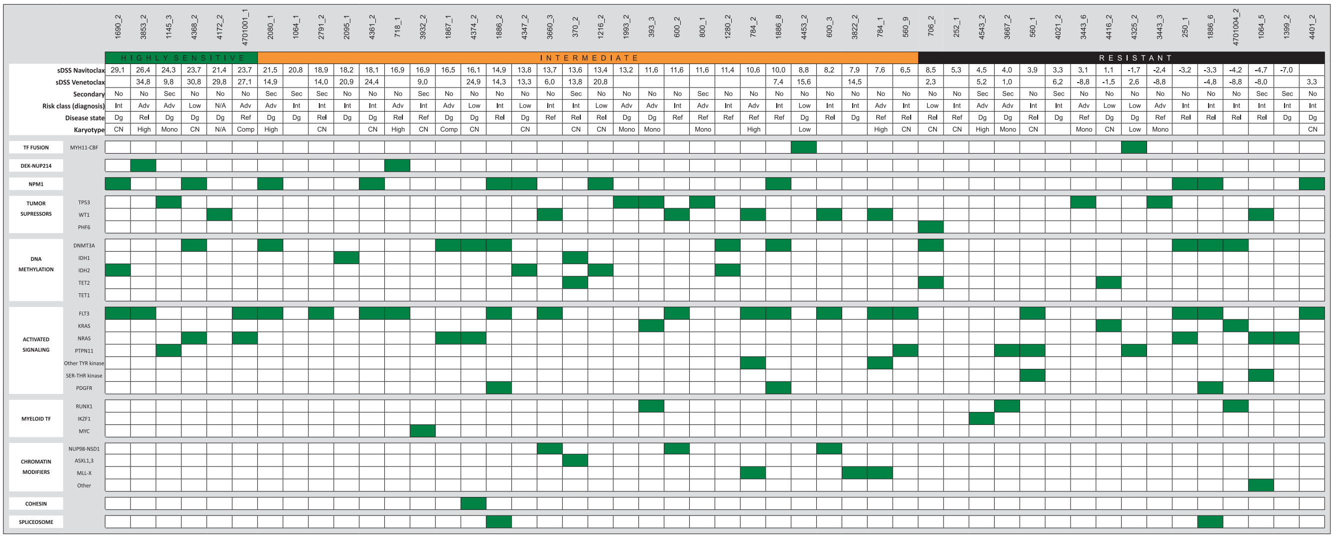

Figure 4. BCL-2 inhibitor responses according to mutational subgroup and disease characteristics. The responses are clustered to highly sensitive, intermediate and resistant. European LeukemiaNet (ELN) risk class[1] is evaluated at the time of diagnosis. The karyotype is presented if examined at the same timepoint with the sampling, a complete list of karyotypes in Supplementary Table 1. All monosomal karyotypes presented as a part of complex karyotype. Adv, adverse risk; CN, normal karyotype; Comp, complex karyotype; Dg, diagnosis; High, high-risk karyotype; Int, intermediate risk; Low, low risk; Mono, monosomal karyotype; Ref, refractory; Rel, relapse; Sec, secondary AML.

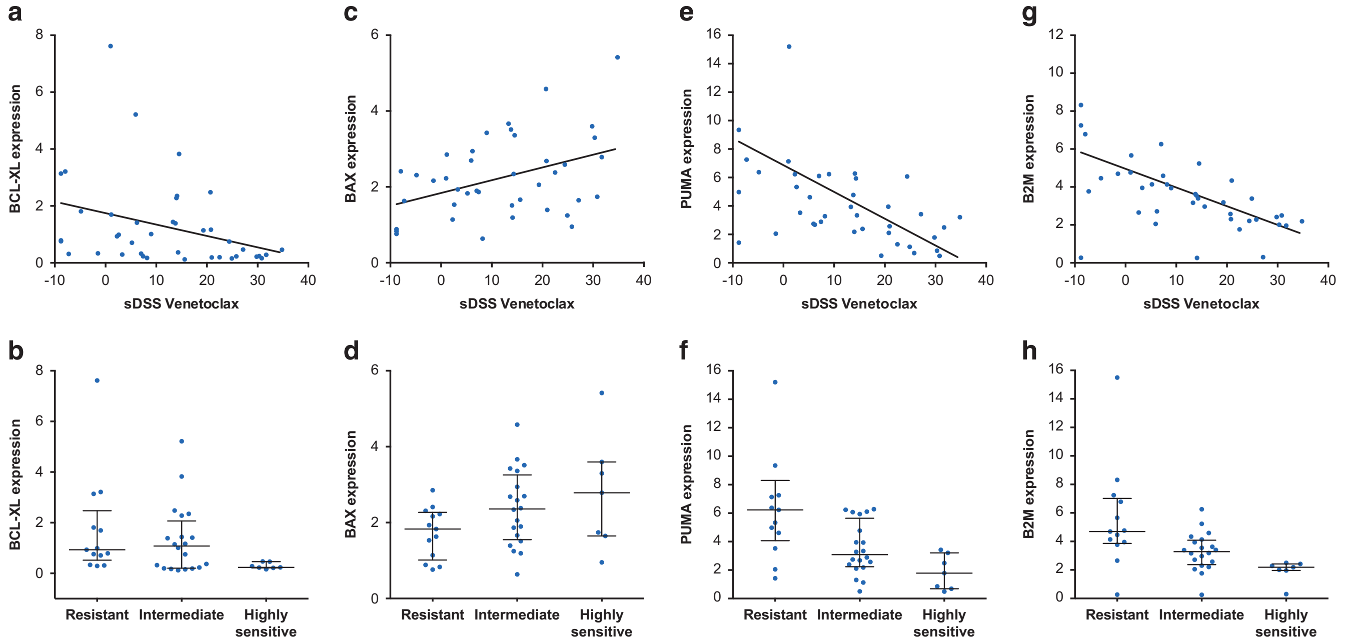

Figure 5. The correlation of transcript level of specific genes to venetoclax sensitivity. (**a**) Anti-apoptotic *BCL-XL* expression and venetoclax sensitivity have an inverse correlation (Spearman's $r = -0.46$, $P = 0.003$). (**b**) *BCL-XL* expression in different response groups (resistant samples, samples with intermediate response and highly sensitive samples). (**c**) Pro-apoptotic *BAX* expression and venetoclax sensitivity have a positive correlation (Spearman's $r = 0.32$, $P = 0.045$). (**d**) *BAX* expression in different response groups. (**e**) Pro-apoptotic *PUMA* has an inverse correlation to venetoclax sensitivity. Spearman's $r = -0.59$, $P < 0.0001$. (**f**) Low expression of *PUMA* was observed in highly sensitive group. (**g**) Correlation was observed between high transcript level of *B2M* and resistance (Spearman's $r = -0.63$, $P < 0.0001$). (**h**) *B2M* expression in different response groups. In (**b**, **d**, **f** and **h**) medians and interquartile ranges are shown.

the BCL-2 inhibitor response. Based on our *ex vivo* data, responses were observed across all cytogenetic groups including complex and monosomal karyotype as well as normal karyotype, and in a similar proportion of samples from diagnosed and relapsed/refractory patients. Responses to venetoclax and navitoclax were similar, but the BCL-2-selective venetoclax exhibited milder responses in control MNCs than the dual BCL-2/BCL-XL inhibitor navitoclax, in line with the clinical experience from these drugs. Venetoclax responses could be divided in three groups: resistant, intermediate and highly sensitive ('CLL-like') samples. Out of 47 samples, 7 (15%) clustered in the highly sensitive group, whereas 15 (32%) were resistant. In concord, in a phase II trial

(NCT01994837) venetoclax monotherapy resulted in complete remissions or complete remission with incomplete blood recovery in 6/32 (19%) of relapsed/refractory AML patients.[26]

BCL-2, BCL-XL and MCL-1 protein levels have previously been correlated to venetoclax sensitivity in AML cells.[14] We observed no correlation with anti-apoptotic *BCL-2* or *MCL1* mRNA expression and venetoclax response. On the other hand, low expression of anti-apoptotic *BCL-XL* and elevated expression of pro-apoptotic *BAX* were associated with sensitivity. Unexpectedly, we also observed inverse correlation of two pro-apoptotic mRNAs, *BIM* and *PUMA*. However, as several BCL-2 family members are regulated by both post-transcriptional and post-translational modifications, it is expected that the mRNA expression level of *BCL-2* family genes—without data on protein levels, especially for known mediators of resistance as MCL-1—insufficiently reflects the vulnerability to venetoclax inhibition.

The observation of high mRNA expression of *B2M* in venetoclax-resistant samples is noteworthy, in particular because B2M protein is a well-established biomarker in lymphatic malignancies.[27] A generally accepted hypothesis has been that serum B2M level correlates to tumor burden, as free soluble B2M is released from the cytoplasm or cell membrane, thus relating to turnover rate of malignant cells.[27] In our cohort, no correlation between venetoclax sensitivity and factors associated with high proliferation in AML, lactate dehydrogenase or leukocyte count was observed. The accumulating data from solid tumors suggest that high expression of *B2M* is independently linked to poor prognosis and that B2M is also involved in proliferation, cell survival and metastasis in various types of cancer.[28–30] In AML and MDS the high serum levels have been shown to correlate to inferior outcome but no further studies on B2M expression or its role in pathogenesis have been carried out.[31,32] Previously, cell line studies have shown B2M to inactivate BAD[33] and to have an effect on apoptosis regulation through increased reactive oxygen,[34] both having links to BCL-2 proteins. The precise effects of B2M on the apoptotic equilibrium and mechanistic explanation for the link to BCL-2 resistance require further investigation.

Our study showed aberrations in chromatin modifiers, *WT1* and *IDH1/2*, to associate with sensitivity to BCL-2 inhibition. *WT1* and *IDH1/2* mutations have been described to result in similar biological effects; *WT1*-mutant AML patients have decreased 5-hydroxymethylcytosine levels consistent with reduced *TET2* function similar to *TET2*/*IDH1*/*IDH2* mutant AML.[35] However, *IDH1* and *IDH2* mutations induce venetoclax sensitivity by 2-hydroxyglutarate-mediated inhibition of the activity of COX in the mitochondrial electron transport chain.[15] Suppression of COX activity consequently lowers the mitochondrial threshold and sensitizes blasts to BCL-2 inhibition, thus possibly presenting a different mechanism of sensitivity than in *WT1*-mutated AML.

Previously, AML cell lines harboring MLL fusion genes have been shown to be sensitive to venetoclax, suggesting MLL fusion genes to have a common role in BCL-2-mediated survival.[36] In this study, samples with aberrations in chromatin modifiers, that is, *MLL*-fusions, *NUP98-NSD1* translocations and *ASXL1* mutation, all exhibited sensitivity to BCL-2 inhibition. These genetic aberrations are associated with elevated *HOX* gene expression; *ASXL1* loss results in a genome-wide reduction in H3K27 trimethylation and *MLL-AF9*, *-AF6* or *NUP98-NSD1* fusion results in DOT1L-mediated hypermethylation of H3K79, with both these events leading to concomitant *HOX* gene expression.[37,38] The potential association to venetoclax sensitivity has been recently explored in acute lymphatic leukemia with MLL-AF4 fusion. The DOT1L-mediated H3K79 methylation results in *BCL-2* overexpression, but not other BCL-2 family members, thus sensitizing cells to venetoclax.[39] Similarly, in HOXA9-dependent leukemia, maintenance of BCL-2 expression has been described to be critical for immortalization and proliferation of leukemic cells.[40]

We detected a distinct *HOX* gene expression signature in sensitive samples. Correspondingly, we observed lack of or low *HOX* gene expression in the resistant samples. Remarkably, a broad-spectrum overexpression of both *HOXA* and *HOXB* genes was detected in samples exhibiting the highest sensitivity, whereas in samples exhibiting intermediate sensitivity, the expression profile was more limited. In human hematopoiesis, *HOX* gene expression is largely restricted to hematopoietic stem cells and progenitor cells.[41,42] Correspondingly, in AML, *HOX* expression is highly regulated.[43] Intriguingly, BCL-2 inhibition has been shown to efficiently induce apoptosis in progenitor cells of high-risk myelodysplastic syndromes and secondary AML patients.[25] Thus, high *HOX* gene expression may characterize a stem/progenitor cell-like AML subgroup that is sensitive to targeted BCL-2 inhibition with venetoclax.

TP53-mutated AML is typical for secondary AML and is associated with chemoresistance.[44] Notably, responses were observed in 4/6 *TP53*-mutated/deleted samples, in concordance with recent CLL studies also showing responses in *17p*-deleted patients.[5] As AML patients with *TP53* mutation currently lack treatment options, BCL-2 inhibitors should be considered for clinical trials in this subgroup. It is also important to recognize that all mutations predicting response to BCL-2 inhibition are among AML-initiating mutations. Targeting post-onset driver mutations (like *FLT3-ITD* and *RAS*) has led to only short-lived responses, perhaps because of the inability to target disease-initiating mutations.[45]

To conclude, we observed venetoclax responses in all disease states of AML, also in relapsed and refractory patient samples. We found several new factors predicting sensitivity to BCL-2 inhibition. Mutations of *IDH1*, *IDH2*, *WT1* and chromatin modifiers predicted selective response to BCL-2 inhibition, whereas *B2M* was the best mRNA-level indicator for anti-BCL-2 drug efficacy. Importantly, we observed that a specific *HOX* gene expression signature was a robust biomarker for venetoclax sensitivity *ex vivo*. Our results can be utilized for identifying BCL2 inhibitor-sensitive AML subgroups for validation in ongoing and upcoming clinical trials.

CONFLICT OF INTEREST

CAH has received research funding from Celgene and Pfizer; BTG has received research funding from Boehringer-Ingelheim Norge AS, and has been on advisory board for BerGenBio AS, Pfizer, Ariad, and Roche; MH has been on advisory boards for Janssen-Cilag and Akinion Pharmaceuticals; KP has received research funding from Celgene; and KW has received research funding from Pfizer.

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AUTHOR CONTRIBUTIONS

MK designed the study, analyzed the data and wrote the manuscript; AK and SE performed sequence data analysis; MMM, TP and JS performed drug sensitivity testing and corresponding analysis; BY and DM performed DSS/sDSS analysis; AP designed and performed the RQ-PCR experiments and analyzed the data; MK, BTG, MH, YF, KR and KP collected the patient specimens and corresponding clinical data; and CH, KW and KP conceived the study, supervised the work and wrote the manuscript. All authors contributed to and approved the final manuscript.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)