Characterization of relapsed Acute Myeloid Leukemia

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Chapter 6

Harnessing Gene Expression Profiles for the Identification of Drug Resistance Genes in Pediatric AML.

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Abstract

Novel strategies are needed to overcome chemoresistance in AML and to improve outcome. To identify genes that are implicated in drug resistance, we correlated exvivo drug resistance data of 73 primary pediatric AML samples to genome wide microarray gene expression profiles of AML blasts obtained at initial diagnosis. We found that ex vivo resistance of AML blasts towards cytarabine (Ara-C), daunorubicin (DNR), etoposide (VP16) and cladribine (2-CdA) correlated significantly with the expression of 87, 377, 504, and 188 genes, respectively. Pathway analyses indicated that Ara-C resistance correlated with chromatin remodeling, epigenetic regulation of gene expression and methyltransferase activity; genes involved included *MLL2*, *MLL4*, *ASXL1*, and *CARM1*. Increased resistance towards VP16 correlated with a low expression of genes that are implicated in cell cycle, DNA replication and DNA damage response (e.g. *BRCA1* and *TOP2A*). A common factor in the correlation of ex vivo resistance towards DNR, VP16 and 2-CdA was the association of the gene expression signature with the *CD40* pathway. We identified gene expression signatures that correlate with ex-vivo drug resistance in pediatric AML. These data may pave the way for the identification of genes that contribute to drug resistance in AML and may subsequently allow its modulation or circumvention.

Introduction

The vast majority of pediatric acute myeloid leukemia (AML) patients achieve complete remission (CR) with current intensive chemotherapy protocols[1]. However, even with optimal induction therapies, 30%--40% of patients relapse[2,3]. Although 65% of these patients achieve a second complete remission (CR2), their 5 year overall survival is 36% at best in latest study protocols[4–6]. The main cause for poor outcome in pediatric AML patients is insufficient treatment that fails to eradicate all leukemic cells.

The backbone of chemotherapeutic regimens in AML contains the deoxynucleoside analogue cytosine arabinoside (Ara-C) that is usually combined with an anthracycline such as daunorubicin (DNR) and often with the topoisomerase inhibitor Etoposide (VP16)[7]. In adult AML patients, a high ex vivo resistance towards Ara-C is associated with an increased risk of relapse and a poor outcome[8]. In pediatric AML, the association of ex vivo drug resistance data with clinical outcome is disputable, since different groups have shown such associations[9,10], whereas others were not able to confirm this[11,12]. The discrepancies in these observations may be due to differences in the contribution of cellular and non-cellular resistance mechanisms in individual patients, such as caused by differences in pharmacokinetics.

Previously, it was shown that patients with a high ex vivo drug resistance towards Ara-C, often are cross resistant, not only towards other deoxynucleoside analogues (e.g. fludarabine or gemcitabine)[13], but also towards other commonly used chemotherapeutics with a different mode of action (such as VP16 or DNR)[14]. Intrinsic properties of leukemic cells that are thought to play an important role in resistance to Ara-C concern for example the ability to transport the drug across the plasma membrane in both directions[15]. Influx of Ara-C may be influenced by the expression of nucleoside transporter proteins that limits drug accumulation at standard dose Ara-C, e.g. via the *SLC29A1* equilibrate nucleoside transporter protein (*hENT1*)[16,17]. On the other hand, effectiveness of other drugs may also be hampered by efflux, which is strongly influenced by the activity of multi-drug transporter proteins, such as MDR1 and other ABC family proteins[18,19]. Besides these characteristics that determine cytoplasmic drug concentrations and retention, other drug resistance mechanisms include alterations in cellular metabolism, proliferative capacity or molecular alterations in the drug target[20].

While most studies focus on individual mechanisms, a few studies have been performed that identify gene expression profiles that associate with drug resistance in younger AML patients. Recently, Lamba et al.[21] performed an integrative analysis of genome wide gene expression data of primary AML cells, ex vivo Ara-C resistance and clinical response parameters (e.g. morphological response, EFS). They found a set of markers that were predictive for beneficial (240 probe sets) or detrimental (97 probe sets) Ara-C related clinical response. Pathway analysis revealed the involvement of the PI3K pathway and *RAS* signaling and suggested an altered proliferation and cell cycle status as a mechanism to escape from chemotherapy.

However, detailed knowledge on the cellular mechanisms that are involved in overall drug resistance towards commonly used chemotherapeutics in AML is still lacking and remains crucial for the development of new treatment strategies that overcome resistance to conventional drugs, eradicate all leukemic cells and ultimately improve outcome in pediatric AML patients. To gain more insight in the cellular properties of leukemic cells that contribute to resistance to four different drugs, we correlate gene expression profiles of AML blasts to their ex vivo drug resistance characteristics using the well-established calorimetric MTT cytotoxicity assay.

Materials and methods

Patients

The current study group consisted of 73 pediatric AML patients from whom we were able to generate both gene expression microarray and ex vivo drug resistance data. The samples were part of a previously published gene expression data set that was used to classify pediatric AML patients[22]. The study was approved by the Institutional Review Board according to national law and regulations and informed consent was obtained for all patients. The gender of 45 out of 73 patients, 79% of the patient group was male, the median age at presentation was 9.3 years and patients belonged to favorable, intermediate and poor cytogenetic risk groups. Patients with MLL-rearranged AML were overrepresented when compared with larger reference pediatric AML groups. Clinical patient characteristics are summarized in Table 1. Viably frozen bone marrow or peripheral blood samples were provided by the Dutch Childhood Oncology Group (DCOG) and the 'Berlin-Frankfurt-Münster' AML Study Group (BFM-AML SG).

Blast enrichment

Leukemic cells were isolated by sucrose density gradient centrifugation and non-leukemic cells were eliminated as previously described[23]. All processed samples finally contained more than 80% leukemic cells, as determined morphologically using cytospins stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). Subsequently, a minimum of 5x106 leukemic cells were lysed in Trizol reagent (Invitrogen, Life Technologies, Breda, The Netherlands). Genomic DNA and total RNA were isolated according to manufacturer's protocols[22]. The remaining viable cells were used for ex vivo drug sensitivity testing using the MTT assay.

Cytogenetics

Leukemic samples were routinely analyzed for cytogenetic aberrations by standard chromosome-banding analysis, and screened by participating childhood oncology group for recurrent non-random genetic aberrations characteristic for AML, including MLL-rearrangements, inv(16), t(8;21) and t(15;17), using either RT-PCR and/or fluorescent in situ hybridization (FISH).

Ex vivo drug sensitivity testing using the MTT assay

Ex vivo cytotoxicity of the deoxynucleoside analogues 1-β-D-arabinfuranosylcytosine (Ara-C, Cytosar; Pharmacia &Upjohn, Woerden, The Netherlands) and 2-chlorodeoxyadenosine (2-2-CdA, Leustatin, Ortho Biotech, USA), anthracycline daunorubicin (DNR, Cerubidine, Rhône-Poulenc, France) and topoisomerase II inhibitor etoposide phosphate (VP16, etoposide-TEVA; TEVA-Pharma, Netherlands) was determined using the 4 days colorimetric MTT assay as described previously[24]. Briefly, six concentrations of each drug were used in the following ranges: Ara-C (0.0098 - 10.0 µg/mL); 2-2-CdA (0.0004 - 40.0 µg/mL); DNR (0.002 - 2 µg/mL), and VP16 (0.05 - 50 µg/mL). Cells without any drug added were included as controls and wells containing culture medium only were used as blanks. The plates were cultured for 4 days at 37°C in a humidified atmosphere containing 5% CO2, after which 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazoliumbromide (MTT; 5 mg ml-1, Sigma Aldrich, Zwijndrecht, The Netherlands) was added. Formazan crystals (indicating metabolically viable cells) were dissolved using acidified isopropanol (0.04 N HCl-isopropyl alcohol) and the optical density (OD) was measured spectophotometrically at 562 and 720 nm. Drug resistance was expressed as the LC_{50} value, the drug concentration achieving 50% lethality of the leukemic cells. Evaluable results were obtained when a minimum of 70%

leukemic blast cells was present at day 4 in control wells and when the control OD was > 0.050.

Microarray analysis

Integrity of total RNA was evaluated using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, USA). cDNA and biotinylated cRNA was synthesized and hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, USA) according to the manufacturer's guidelines. Arrays with poor quality were excluded from further analysis as recommended by the manufacturer.

Data preprocessing

We applied the variance stabilization normalization procedure (VSN25) to remove background signal and normalize raw data across arrays. Log2 transformed expression values were calculated from perfect match (PM) probes only and summarized using a median polish method. The original and processed data have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under GEO Series accession number GSE17855.

Correlation of microarray data with LC_{50} values

To identify gene expression associated with ex vivo drug resistance, we correlated the VSN normalized expression values with the LC_{50} values of the MTT assays using Spearman rank correlation in BRB Arraytools. A nominal P of 0.001 was used to identify significantly correlated genes. In addition, we performed non parametric Global testing26. A schematic overview of analyses is given in Figure 1.

Software

R (version 2.10.1) and the R packages affy, affyQC, simpleaffy, affyPLM, vsn, globaltest, limma, multtest and marray were used to run the abovementioned analyses. Hierarchical clustering analysis with average linkage was performed using Cluster 3.0 and visualized using Treeview. Correlation and Gene Ontology (GO) analyses were performed using BRB Arraytools 4.1. Pathway analysis was performed using Ingenuity Pathway Analysis 7.5 software (Ingenuity Systems, Redwood City, CA, USA) based on the Ingenuity Pathways Knowledge Base.

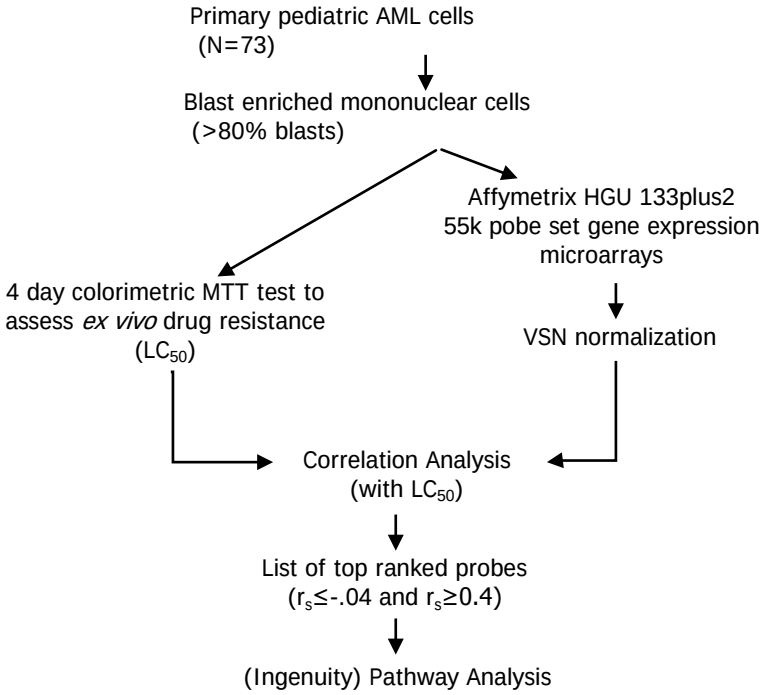

Figure 1. Schematic overview of analyses. Mononuclear cells were isolated from 73 primary pediatric AML samples at initial diagnosis. These mononuclear cells were either tested for ex vivo vitro resistance towards ara-C, DNR, VP16 and CDA or they were used for gene expression microarray analysis. The data from these assays were correlated and on the obtained gene lists for each drug, pathway analysis was performed.

Results

Ex vivo drug resistance of primary AML blasts

We successfully tested primary blast cells of pediatric AML patients for ex vivo resistance towards Ara-C (N=73), DNR (N=69),VP16 (N=39) and 2-CdA (N=59), chemotherapeutic drugs that are currently used to treat AML (excluding 2-CdA) All these drugs showed a dose-dependent cytotoxicity in the tested concentration ranges. The median LC_{50} values are shown in Table 1 and the LC_{50} distribution for all 4 drugs and in the whole patient group is shown in Figure 2. To test whether patients showed drug cross-resistance, we performed a Spearman rank correlation test on LC_{50} values of the four drugs. An overview of the correlations of drug resistance is shown in Supplementary Table 1 (Appendix). Indeed, a marked cross- resistance of patients' blasts was found for VP16 and the other drugs; Ara-C (rs=.43, P=.006) and in particular for DNR and 2-CdA (rs=.63 and rs=.59, P=.00003 and P=.0004, respectively). The cross-resistance of Ara-C and DNR or 2-CdA was weaker, with the strongest correlation coefficient between Ara-C and the other nucleoside analogue, 2-CdA (rs=.44, P=.001).

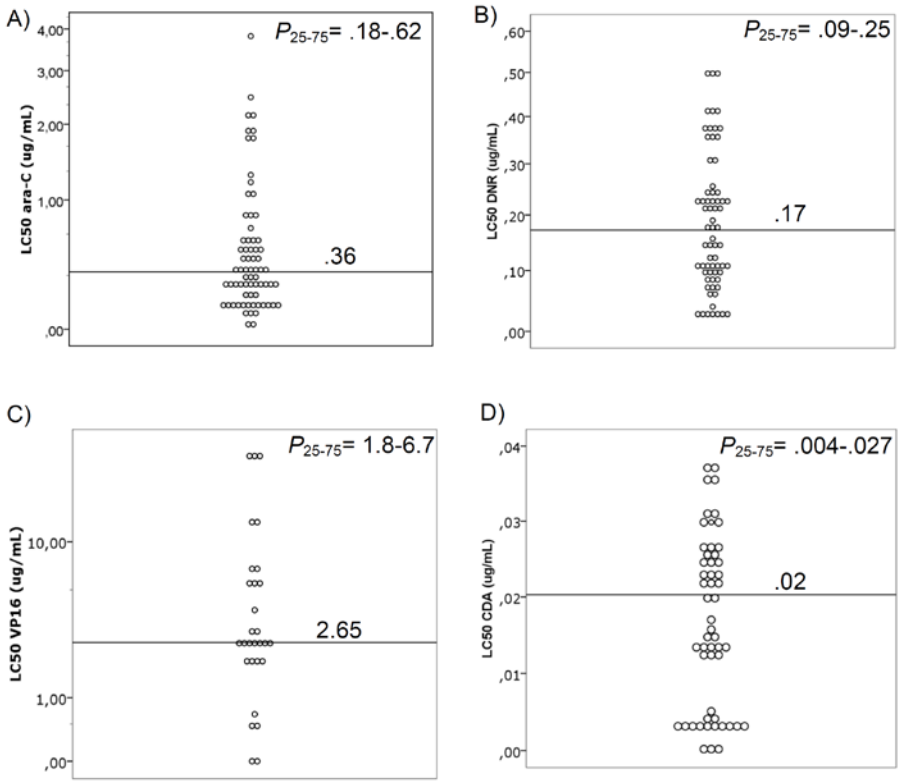

Figure 2. Distribution of ex vivo drug resistance data (LC50) for standard chemotherapeutical drugs. Ex vivo vitro drug sensitivity of primary AML cells to: A) ara-C, B) DNR, C) VP16 and D) CDA. Data are depicted in LC50 values, the drug concentration (ug/mL) at which 50% of the cells in the assay die. Each dot represents the LC50 value of an individual sample. The horizontal line depicts the median LC50 value.

Correlation of gene expression with ex vivo drug resistance

Spearman's rank correlation analyses were used to correlate gene expression to the ex vivo drug resistance data, based on the correlation coefficient and a stringent P value of 0.001 to evaluate statistical significance. For Ara-C, DNR, VP16 and 2-CdA, we found 112, 465, 656 and 269 probe sets, respectively, that were significantly associated with ex vivo drug resistance data. Complete lists are provided in Supplementary Tables 2,3,4 and 5, respectively. The probe sets represent 87, 377, 504, and 188 coding genes, respectively. Figure 3 shows heat maps of the intensity levels of all significantly associated probe sets, ranked according to ex vivo drug resistance (LC_{50} values). Table 2 shows the top ten positively (Table 2A) and negatively (Table 2B) correlating genes for each drug. The ranges of correlation coefficients are shown in Table 3. The strongest correlations between the ex vivo drug resistance and gene expression were found for VP16, the correlation coefficient ranged from rs= -0.78 to 0.69, parametric P values ranged between $1x10^{-4}$ and $2x10^{-7}$.

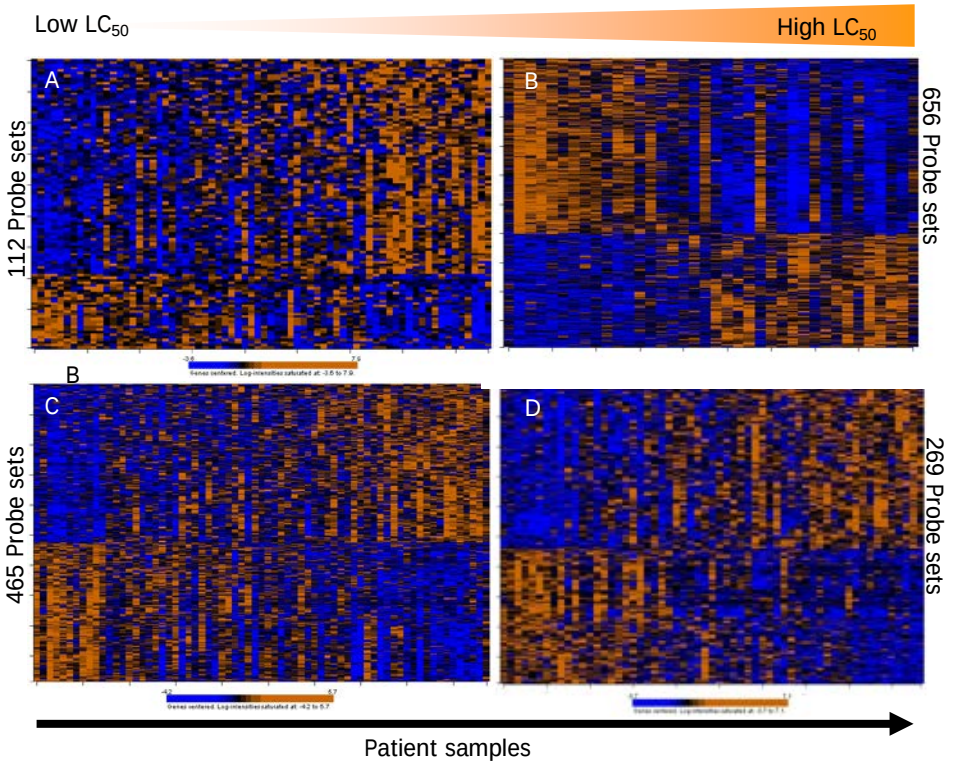

Figure 3. Heatmaps of ex vivo drug sensitivity associated gene expression patterns of pediatric AML blasts. Probe sets (rows) were clustered unsupervised using average linkage and samples were ranked according to in vitro drug sensitivity towards A) Ara-C, B) DNR, C) VP16 and D) CDA. The white to orange color gradient indicates a gradient from ex vivo drug sensitive to ex vivo drug resistant samples respectively. The depicted probe sets for each drug are listed in Supplementary Tables 2-5.

Table 2 Top ten list of genes that are: A) positively and B) negatively correlated with ex vivo Ara-C, DNR, VP16 and 2-CdA resistance, ranked according to the Spearman correlation coefficient

	Ara-C			DNR			VP16			2-CdA		
A	**rs**	**P**	**Symbol**	**rs**	**P**	**Symbol**	**rs**	**P**	**Symbol**	**rs**	**P**	**Symbol**
1	0,51	9,20x10^{-06}	ARFGAP1	0,57	4,00 x10^{-07}	TNFSF8	0,69	3,30 x10^{-06}	MALAT1	0,54	1,18 x10^{-05}	CYTIP
2	0,50	1,42 x10^{-05}	GGA3	0,54	2,80 x10^{-06}	AKAP13	0,64	2,52 x10^{-05}	CPAMD8	0,53	1,91 x10^{-05}	GLS
3	0,50	1,74 x10^{-05}	MBRL	0,53	4,60 x10^{-06}	CLEC7A	0,63	3,35 x10^{-05}	SPATA13	0,53	2,65 x10^{-05}	MALAT1
4	0,49	2,30 x10^{-05}	DCAF15	0,53	4,80 x10^{-06}	RAPGEF3	0,63	3,65 x10^{-05}	CLEC7A	0,52	3,73 x10^{-05}	ZNF706
5	0,49	2,39 x10-^{05}	MLL2	0,52	5,50 x10^{-06}	TNFSF8	0,63	3,76 x10^{-05}	AVPI1	0,51	4,32 x10^{-05}	BNIP3L
6	0,49	2,58 x10^{-05}	VAMP2	0,52	6,90 x10^{-06}	PPFIA1	0,63	4,10 x10^{-05}	RGPD5	0,51	5,45 x10^{-05}	FKSG2
7	0,48	3,90 x10^{-05}	TNPO2	0,51	8,70 x10^{-06}	TNFAIP3	0,62	4,42 x10^{-05}	GRASP	0,50	6,33 x10^{-05}	MEF2A
8	0,47	4,18 x10^{-05}	RHOC	0,51	9,10 x10^{-06}	HAVCR2	0,61	5,81 x10^{-05}	ANGPTL4	0,50	6,35 x10^{-05}	NEAT1
9	0,47	5,66 x10^{-05}	MLL4	0,50	1,35 x10^{-05}	SH2D1A	0,61	5,85 x10^{-05}	SSBP4	0,50	6,40 x10^{-05}	NLRP1
10	0,46	6,26 x10^{-05}	PPM1K	0,50	1,39 x10^{-05}	LRRC36	0,61	6,30 x10^{-05}	CYMP	0,50	6,46 x10^{-05}	OTUD5
B												
10	-0,42	2,99 x10^{-04}	LRRC16A	-0,49	2,09 x10^{-05}	GGCX	-0,65	1,75 x10-05	MCM10	-0,50	7,58 x10^{-05}	BRE
9	-0,42	2,87 x10^{-04}	CKLF	-0,50	2,00 x10^{-05}	COX7A2	-0,66	1,26 x10-05	IMP3	-0,50	5,95 x10^{-05}	PRG4
8	-0,43	2,77 x10^{-04}	PTPMT1	-0,50	1,90 x10^{-05}	PTPMT1	-0,66	1,24 x10-05	PRRC1	-0,51	5,62 x10^{-05}	AKR1A1
7	-0,44	1,61 x10^{-04}	FAM82B	-0,50	1,68 x10^{-05}	COX7A2	-0,66	1,15 x10-05	PCNA	-0,51	5,50 x10^{-05}	ZNF532
6	-0,46	8,57 x10^{-05}	TMEM38B	-0,51	1,23 x10^{-05}	UBE2E3	-0,66	1,04 x10-05	MRPS18C	-0,51	4,81 x10^{-05}	H6PD
5	-0,46	7,20 x10^{-05}	FAM162A	-0,51	1,03 x10^{-05}	EFCAB11	-0,66	1,04 x10-05	UGGT1	-0,51	4,56 x10^{-05}	PTPMT1
4	-0,47	4,19 x10^{-05}	KIAA0564	-0,51	8,80 x10^{-06}	TSN	-0,68	5,10 x10-06	ZHX1	-0,52	3,95 x10^{-05}	LRRC20
3	-0,48	3,64 x10^{-05}	PPA2	-0,53	3,50 x10^{-06}	KCTD15	-0,68	4,60 x10-06	NOP16	-0,53	2,39 x10^{-05}	WHAMML2
2	-0,49	2,35 x10^{-05}	UTS2	-0,56	9,00 x10^{-07}	ALS2CR4	-0,68	4,30 x10-06	FEN1	-0,53	2,11 x10^{-05}	CCL23
1	-0,54	2,20 x10^{-06}	CLCC1	-0,59	2,00 x10^{-07}	PON2	-0,78	2,00 x10-07	HNRNPAB	-0,53	2,04 x10^{-05}	KAZ

rs= Spearman's correlation coefficient; a parametric P of 0.0001 was used to determine significant correlation

Table 3. Overview of Spearman's rank correlation test results of correlation of *ex vivo* drug resistance of primary AML blasts with genome wide gene expression data.

Drug	significant probe sets	range of r_s	parametric range	p-value	permutation range	p-value
Ara-C	113	-0,54 to 0,57	$1x10^{-4}$ to $5.0x10^{-7}$		$1.7x10^{-3}$ to $<1x10^{-7}$	
DNR	465	-0,59 to 0,57	$1x10^{-4}$ to $2.2x10^{-7}$		$1.8x10^{-3}$ to $<1x10^{-7}$	
VP16	656	-0,78 to 0,69	$1x10^{-4}$ to $2.0x10^{-7}$		$1.8x10^{-3}$ to $<1x10^{-7}$	
CDA	269	-0,53 to 0,58	$1x10^{-4}$ to $3.0x10^{-6}$		$1.4x10^{-3}$ to $<1x10^{-7}$	

Since we observed both correlation between LC_{50} of individual drugs with gene expression and drug cross resistance of primary AML blasts, we hypothesized that certain gene expression profiles may correlate with an overall ex vivo drug resistance including several different drugs. Therefore, we assessed the overlap between the lists of probes that correlated with ex vivo resistance data of the individual drugs. A total of 76 genes and two validated open reading frames overlapped between two or more of the drugs analyzed. A schematic overview is provided in Figure 4, and a complete overview of overlapping probe sets is provided in Supplementary Table 6. An overlap of drug resistance associated genes (a total of 36 genes) was most frequent for VP16 and DNR. Twelve genes correlated with both VP16, DNR and 2-CdA resistance. When data of all four drugs studied were combined, no overlap was observed. Moreover, no overlap was found between Ara-C and DNR.

We then hypothesized that similar pathways may be mediating or contributing to the drug resistance observed ex vivo. To determine which biological pathways were associated with ex vivo drug resistance according to the correlated gene expression profiles, we performed Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) on the probe sets that correlated with ex vivo resistance to the four drugs (listed in Supplementary Tables 2-5).

As an example, elevated expression levels of *MLL2*, *MLL4*, *ASXL1*, and *CARM1* were associated with high LC_{50} values of Ara-C (Supplementary Table 2). The 112 probe sets that were associated with ex vivo Ara-C resistance were dispersed over a number of pathways with relatively low Ps, ranging from $8.1x10^{-3}$ to $4.42x10^{-02}$.

This included several pathways that were involved in membrane integrity and membrane signaling. Chromatin remodeling, epigenetic regulation of gene expression and methyltransferase activity were among the top GO categories for Ara-C resistance correlated gene expression patterns. IPA predicted that the observed gene expression profiles may be associated with upstream regulation by IL5 (Table 4).

IPA on the genes, the expression of which was correlated with ex vivo resistance of AML blasts towards DNR (Supplementary Table 3), showed enrichment in canonical pathways that mediate signaling in cell-to-cell interaction, apoptosis, antigen presentation in response to growth factor signaling or oxidative stress (P values ranged from $1.3x10^{-2}$ to $3.3x10^{-3}$, Table 4). We used IPA again to predict factors that could explain the observed gene expression patterns and the top five upstream regulators were: *CD40L*, *IRF8*, *OSCAR*, *TNF*, *POLR2A* (see Supplementary Table 7 for a complete list).

Gene expression that correlated to ex vivo resistance of AML blasts towards VP16 (Supplementary Table 4), was associated with GO categories and canonical pathways that involved cell-to-cell signaling, histone modification, cell cycle and DNA damage response (Table 4). The most important genes that are implicated in these cellular processes are shown in Table 2. Similar to what was found for the ex vivo resistance towards DNR, *CD40L* was identified among the upstream regulators of VP16 resistance associated gene expression patterns. Other important upstream regulators according to

IPA were *BRCA1*, *ACAT1*, *STX12* and *NR1H3*, a complete list is provided in Supplementary Table 7.

Among the GO categories and pathways that overlapped with the gene expression patterns that correlated with 2-CdA ex vivo resistance (Supplementary Table 5) were several cellular metabolism associated pathways, for example, aldehyde metabolic processing or glutamine metabolism. Other pathways were, for example, related to receptor tyrosine kinase activity or immune response signaling (Table 4). IPA analysis pointed to the following upstream regulators that may explain the observed 2-CdA resistance associated gene expression patterns: *CD40L*, *ASB2*, *IL10RB*, *RUNX1* and *IL2* (Supplementary Table 6).

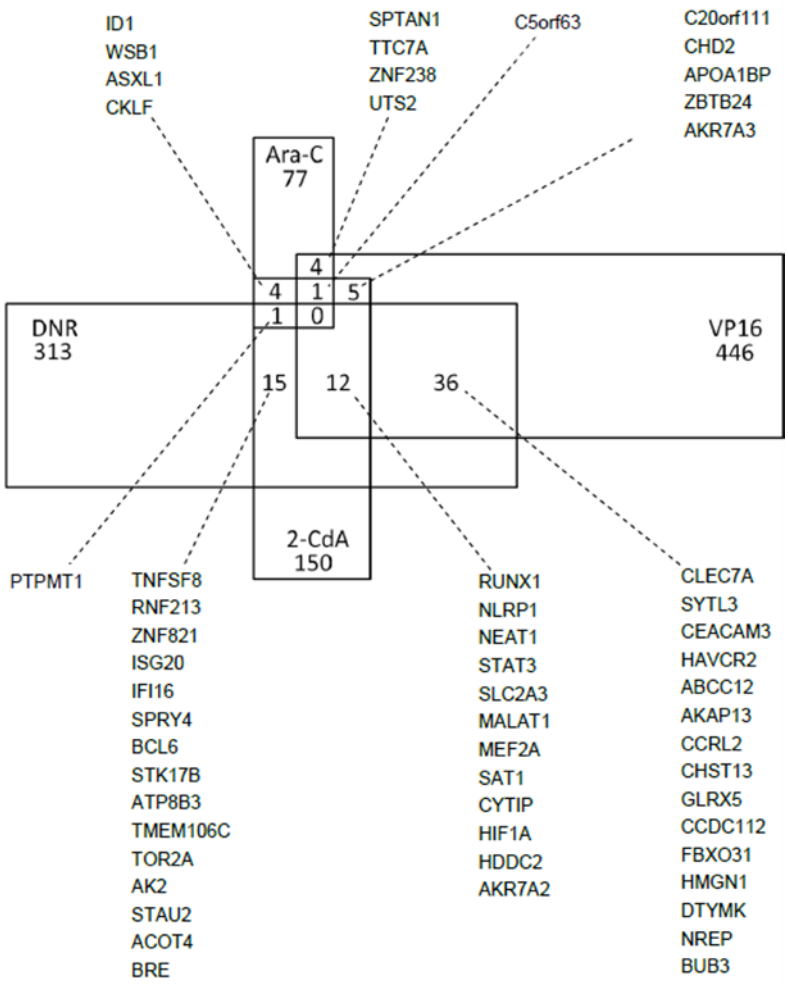

Figure 4. A schematic depiction of the number of genes that correlate significantly with ex vivo drug resistance in a single or two or more of the studied drugs (Ara-C, VP16, DNR, 2-CdA). Overlapping genes are shown, ranked according to Spearman rank correlation coefficient. For the 36 genes that overlapped between DNR and VP16, the top fifteen correlating genes are shown

Table 4 Summay of pathway analysis on gene expression that correlated with ex vivo drug resistance tests

Drug	top 3 GO categories	top 3 molecular pathways	top 3 upstream regulators	*P* range upstream regulators
Ara-C	Chromatin remodeling	Biosynthesis of Steroids	IL5	$2.40x10^{-02}$
	Epigenetic regulation of gene expression	HMGB1 Signaling		
	Gene silencing	Semaphorin Signaling in Neurons		
DNR	Cellular response to growth hormone stimulus	Growth Hormone Signaling	CD40L, IRF8, OSCAR	$7.4x10^{-4}$ to $4.4x10^{-5}$
	Growth hormone receptor signaling pathway	Oxidative Phosphorylation		
	Response to growth hormone stimulus	IL-9 Signaling		
VP16	Protein-arginine N-methyltransferase activity	RAN signaling	CD40L, BRCA1, ACAT1	$3.2x10^{-2}$ to $9.6x10^{-3}$
	Histone lysine demethylation	Role of OCT4 in Mammalian Embryonic Stem Cell Pluripotency		
	Histone demethylation	Role of CHK Proteins in Cell Cycle Checkpoint Control		
CDA	Cellular aldehyde metabolic process	D-glutamine and D-glutamate Metabolism	CD40L, ASB2, IL10RB	$1.15x10^{-2}$ to $8.3x10^{-4}$
	Transmembrane receptor protein serine/threonine kinase activity	Polyamine Regulation in Colon Cancer		
	Coated pit	IL-22 Signaling		

GO=Gene Ontology, molecular pathways and upstream regulators are ranked according to p-value

Discussion

In the current study we aimed at identifying mechanisms that may confer drug resistance upon AML blast cells. We therefore correlated gene expression patterns with ex vivo resistance of primary leukemic blasts towards three chemotherapeutic agents, which are commonly used in the treatment of AML (Ara-C, DNR and VP16). We performed drug resistance testing and genome wide gene expression profiling in a large group of 73 pediatric AML patients (Table 1). For each drug the expression of a different set of genes significantly correlated with ex vivo resistance. Although not part of standard chemotherapeutic regimens, 2-CdA was tested as second nucleoside analogue[27,28] and indeed 2-CdA resistance correlated significantly with Ara-C resistance.

Correlation of gene expression profiles with ex vivo drug resistance of Ara-C yielded many interesting genes with implications in cancer (Supplementary Table 2). However, we did not find evidence for deregulated gene expression of any of the known key players in metabolism or transport of Ara-C. Gene ontology analysis did show that the genes that were associated with ex vivo Ara-C resistance were mainly involved in chromatin organization and epigenetic gene regulation. Individual genes implicated in these categories are for example *CARM1*, *ARID1A*, *ASXL1*, *MLL2* and *MLL4*. They act primarily by altering the histone landscape. The mixed lineage leukemia (*MLL*) family genes encode for a set of conserved proteins that have a histonemethyltransferase activity, which allows regulation and activation of gene transcription, e.g. *HOX* genes. They exert different cellular functions and are involved, amongst others, in mRNA processing, nuclear hormone receptor binding or DNA damage response[29]. In pediatric AML, *MLL* genes are notorious because of the frequent rearrangements that comprise a cytogenetic subgroup of often poor risk patients30. It was previously shown that cellular drug sensitivity of *MLL*-rearranged AML depends on the fusion partner of the 11q23 arm31 and in concordance, e.g. t(9;11) patients were less resistant to Ara-C in our study (data not shown). However, patients lacking chromosome 11 aberrations displayed a similar signature that was associated with ex vivo drug resistance, including MLL2/4 expression levels. Therefore, increased wild type MLL gene expression may also play a role in cellular resistance towards Ara-C. In cancer, wild type MLL genes play a crucial role in eukaryotic transcription factor 2 (*E2F1*) mediated DNA damage response and apoptosis. E2F1 associates with MLL family ofhistone methyltransferases and with host cell factor 1 (*HCF1*) to allow efficient DNA damage repair. The activity of *HCF1* is regulated by host cell factor C1 regulator 1 protein (HCFC1R1)[32], the expression of which was also positively associated with Ara-C resistance ($rs=.394$, $P= 8x10^{-4}$).

In recent years, an increasing number of mutations in genes regulating epigenetics have been identified which are considered to play a crucial role in myeloid neoplasia, including AML[33,34]. Besides a putative role in leukemogenesis, such mutations may provide strong adverse prognostic factors in adult AML, e.g. *DNMT3A*[35] or *ASXL1*[36,37]. Hence, therapeutics that target chromatin modifications are currently of great interest as novel targeted treatment strategies. Although mutations in epigenetic modifiers are infrequent in pediatric AML[38,39], the prevalence of such genes in our Ara-C drug resistance signature, suggests that epigenetic routes may play a role in drug resistant pediatric AML and these patients may therefore benefit from drugs that target chromatin modifications.

The most frequent overlap of genes that correlated with drug resistance was observed for DNR and VP16. For these drugs, that both interfere with DNA replication also via inhibition of topoisomerase IIa (*TOP2A*)[40], the expression correlated with drug resistance particularly with genes implicated in the mechanism of action of these drugs; for example, the gene expression levels of DNA polymerases *POLE3* and *POLE4* were inversely correlated to DNR resistance. In concordance with previous reports[40], *TOP2A*

gene expression -the primary direct target of VP16- was inversely correlated with ex vivo VP16 resistance (rs=0.54, P=5.6x10^{-4}). We found that molecules that are implicated in drug transport correlated with drug resistance for more than one of the drugs. For example, the expression of multidrug resistance protein 9 (*MRP9*/*ABCC12*) correlated with ex vivo drug resistance to DNR and VP16 (rs=.40 and rs=.40, P=6.3x10^{-4} and P=9.8x10^{-4} respectively, Supplementary Table 5). Although the role of this specific ABC transporter remains elusive, it is highly expressed in breast cancer patients[41] and it shows a high degree of similarity with MRP5, which is implicated in nucleotide and nucleotide analogue export[42]. In addition, the expression of 2, 4, 12 and 6 members of the solute carrier family proteins (SLC) were associated with ex vivo resistance towards Ara-C, DNR, VP16 and 2-CdA respectively (Supplementary Tables 2-5). SLC proteins comprise a large family of transporter molecules, some of which are directly or indirectly associated with drug resistance. For example, in our data set, *SLC2A3* (*GLUT3*) expression was positively correlated with drug resistance towards DNR, VP16 and 2-CdA (rs=.47, rs=.59, rs=.48 and P=5.6x10-5, P=1.4x10-4, P=1.3x10-4 respectively, Supplementary Tables 3-5). In murine erythroid leukemia cells, the glucose transport inhibitor cytochalasin B that binds to *SLC2A3* was shown to directly inhibit MRP-mediated drug efflux[43]. *SLC2A3* expression levels were found to be increased in virtually all types of cancer cell lines, including leukemia. In these cell lines it was also shown that the use of glucose transport inhibitors that interact with *SLC2A3* and related molecules can sensitize cancer cells for drugs such as DNR under hypoxic conditions[44].

Pathway analyses using the gene expression signatures of drug resistance towards each cytotoxic agent showed that for three of the four drugs studied (DNR, VP16 and 2-CdA), the gene expression signature may be the result of an upstream regulation by CD40 ligand (*CD40L*) signaling. The CD40 pathway is implicated in several types of cancer[45], including a number of hematological malignancies[46]. Constitutive activation of the CD40 pathway may promote tumor cell proliferation and survival[47]. This may be the case for AML as well, since high levels of soluble CD40L can be observed in the serum of multiple myeloma (MM) and AML patients[48]. In MM cells, CD40 stimulation using recombinant human CD40L was shown to directly promote drug resistance by enhancing *MDR1* (ABCC1/P-glycoprotein) expression[49]. Its diverse roles in various cancer types makes the CD40 pathway an attractive target for therapy and therapeutics have been developed. Some of these therapeutics have shown in vitro and in vivo activity and/or synergistic effect with standard chemotherapeutics[47,50], yet the benefit of targeting CD40 in AML remains elusive.

In summary, in the current study we present novel data in which diagnosis samples of a large group of pediatric AML patients were used to identify gene expression profiles that are associated with ex vivo cellular drug resistance. We found gene expression signatures of ex vivo resistance towards standard anti-leukemic drugs that implicate both classical cellular drug resistance pathways (e.g. drug efflux) as well as pathways that are related to cell growth, oxygenation and energy metabolism and tumor (micro-) environment-related mechanisms. These observations enhance the notion that multiple deregulated pathways may be required to acquire a multidrug resistance phenotype in myeloid neoplasia[51]. Our data indicate that epigenetic mechanisms are also implicated in drug resistance. Epigenetic aberrations, together with genetic lesions may underlie gene expression signatures that contribute to chemoresistance[52–54]. These mechanisms may synergistically confer drug resistance[55] upon cells that survive therapy after initial treatment thereby resulting in relapse of AML[56,57]. Our current data may pave the way towards the identification of genes that contribute to drug resistance, such as *CD40L*. Moreover, our findings may enhance the development of personalized treatment strategies by individualized approaches to modulate or circumvent resistance to conventional chemotherapeutic drugs.

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