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Bachas, C.

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Chapter 5

Gene expression profiles that are associated with relapse of pediatric AML.

Costa Bachas, Gerrit Jan Schuurhuis, C. Michel Zwaan, Marry M. van den Heuvel-Eibrink, Monique L. den Boer, Eveline S. de Bont, Zinia J. Kwidama, Dirk Reinhardt, Ursula Creutzig, Valerie de Haas, Gertjan J.L. Kaspers, Jacqueline Cloos

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Abstract

Development of relapse remains a problem for further improvements in the survival of pediatric AML patients. While virtually all patients show a good response to initial treatment, most patients respond poorly when treated at relapse. The cellular characteristics of leukemic blast cells that allow survival of initial treatment, relapse development and subsequent resistance to salvage treatment remain largely elusive. We aim to assess such characteristics by studying biological differences between diagnosis and relapse samples. Therefore, we performed microarray gene expression profiling on paired initial and relapse samples of 23 pediatric AML patients. In 11 out of 23 patients, gene expression profiles of initial and corresponding relapse samples end up in different clusters in unsupervised analysis, indicating altered gene expression profiles. In addition, shifts in type I/II mutational status were found in 5 of these 11 patients, while 3 shifts were found in the remaining 12 patients. Although differentially expressed genes varied between patients, they were commonly related to hematopoietic differentiation, encompassed genes involved in chromatin remodeling and showed associations with similar transcription factors. The top five were C*EBPA, GFI1*, *SATB1*, *KLF2* and *TBP*. In conclusion, the leukemic blasts at relapse are biologically different from their diagnosis counterparts warranting novel treatment strategies.

Introduction

In high income countries, the vast majority (over 90%) of pediatric acute myeloid leukemia (AML) patients achieve complete remission (CR) with current intensive chemotherapy protocols.[1] However, even with optimal therapies, 30-40% of patients relapse and face a dismal prognosis.[2–4] As a result, long term survival rates have only marginally increased over recent decades and stabilized at approximately 65%.[5]

Poor response in terms of blast reduction at initial diagnosis[6,7] and/or later at relapse[4] is among the strongest adverse prognostic factors for outcome in pediatric AML.[8] Apparently, therapy is insufficient to relieve a quarter of the patients from a burden of persistent leukemia that causes relapses and fatal outcome.

A number of cell-biological factors determined at initial diagnosis are known to be associated with an increased risk of relapse, including genetic characteristics such as aberrant karyotype (e.g. certain 11q23 translocations or monosomy 7)[9,10] or gene mutations such *FLT3/ITD* and *WT1.*[11] Moreover, aberrant gene expression (e.g. *EVI-1*, *BAALC, WT1)* has also been reported to be associated with the risk of relapse.[12,13] The mechanisms by which these factors act in relapse development are largely unclear.

The mainstay of salvage regimens in relapsed AML is, as in initial treatment, cytarabine and anthracycline based,[2,14] hence drug resistance is likely to play a role in relapse development. Although pharmacological factors may be involved in resistance to therapy,[15] cellular drug resistance is thought to contribute to poor response to therapy as well.[16,17] Putative drug resistance mechanisms are for example over-expression of genes that encode drug efflux proteins such as the ATP-binding cassette (ABC) transporter gene *MDR1* or *MRP1* and *LRP* genes.[18] Drug uptake may also be impaired by an altered activity of nucleoside-specific membrane transport carriers, such as *hENT1.*[17] Alternatively, genes that impair apoptosis such as *BCL2* and *BCLX* genes may be upregulated in AML and render leukemic cells insensitive to chemotherapy.[19] Drug resistance may also result from leukemic cells with a quiescent stem cell phenotype that are less sensitive towards the cell cycle dependent chemotherapeutics.[20]

Previous studies with paired initial and relapsed AML samples showed that the mutational status[21], cytogenetics[22,23] and cell surface protein expression[24,25] of AML cells may change during treatment in a large portion of patients (>40%). Such biological differences may be crucial in the development of relapse and this warrants further investigation.

In summary, novel therapeutic strategies that aim to prevent relapse or improve outcome of relapsed AML patients are needed. However, the precise biological background of AML relapse remains largely elusive and more detailed knowledge on the specific characteristics of the relapsed AML cells is required. In this study, we determined differences in genome wide gene expression of corresponding initial and relapse AML samples to find genes and gene expression profiles that play a role in development of relapse. The contribution of mutational shifts to differential gene expression was evaluated and molecules and pathways related to relapse development that were commonly affected in patients were identified.

Materials and methods

Patients

We studied initial and corresponding first relapse samples (N=46) of 23 pediatric AML patients. Viably frozen bone marrow or peripheral blood samples from pediatric AML patients were provided by the Dutch Childhood Oncology Group (DCOG) and the 'Berlin-

Frankfurt-Münster' AML Study Group (BFM-AML SG). The initial diagnosis samples were part of a previously published data set.[26] The study was approved by the Institutional Review Board according to national law and regulations and informed consent was obtained for all patients. Patients who suffered from recurrent disease within 2 years after initial diagnosis were selected. Clinical patient characteristics are summarized in Table 1.

Blast enrichment

Leukemic cells were isolated by sucrose density centrifugation and non-leukemic cells were eliminated as previously described.[27] All processed samples contained more than 80% leukemic cells, as determined morphologically using cytospins stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). Subsequently, a minimum of $5*10^6$ leukemic cells were lysed in Trizol reagent (Invitrogen, Life Technologies, Breda, The Netherlands). Genomic DNA and total RNA were isolated according to manufacturer's protocol.

Cytogenetics

All 23 initial diagnosis leukemia samples and 10 out of 23 relapse samples were routinely investigated for cytogenetic aberrations by standard chromosome-banding analysis, and screened 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) by each study group or, in case of lacking data of initial samples, this was performed specifically for this study.

Mutation analyses

Samples were screened for hotspot mutations in *NPM1*, *CEPBA*, *FLT3*, *NRAS*, *KRAS*, *PTPN11*, *KIT* and *WT1* as previously described.[21]

Gene expression profiling and quality control

Integrity of total RNA was checked 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 according to the manufacturer's recommendations were excluded from further analysis.

Data preprocessing

We applied the variance stabilization normalization procedure (VSN)[28] to remove background signal and normalize raw data across arrays. Log_2 transformed expression values were calculated from perfect match (PM) probes only and summarized using a median polish method. The original and processed data from diagnosis and relapse samples have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under GEO Series accession number GSE17855[26] and GSE45288 respectively.

Statistical analysis

Probes with expression intensity below 30 were excluded from further analysis for previously mentioned reasons.[29] To identify differentially expressed probes in the VSN normalized expression values, we performed significance analysis of microarrays

(SAM).[30] We accepted a maximal false discovery rate (FDR) of 25% of cases with a confidence interval (CI) of 80%. Fold change expression differences of individual probe-sets between two classes (e.g. diagnosis and relapse) were calculated as ratios of geometric means, i.e. the anti-log of the arithmetic mean of the logs of probe-set intensities in each class. For survival analysis we used Kaplan-Meier analysis with log rank testing and Cox regression analysis to calculate the Hazard Ratio (HR) using SPSS version 20. Two sided p values below 0.05 were considered statistically significant.

Software

R (version 2.10.1) and the Bioconductor packages affy, affyQC, simpleaffy, affyPLM, and VSN were used for quality control and preprocessing of raw data. Hierarchical clustering analysis with average linkage was performed using Cluster 3.0[31] and visualized using Gene-E (http://www.broadinstitute.org/cancer/software/GENE-E/). Class comparison tests and class prediction tests were performed using the Biometric Research Branch Arraytools 4.2.1. Pathway analysis was performed using Ingenuity Pathway Analysis 7.5 software (Ingenuity Systems, Redwood City, CA, USA) based on the Ingenuity Pathways Knowledge Base.

Real-time quantitative PCR

The expression data for selected genes were validated in 8 independent AML patient samples using real-time quantitative PCR. The GUS gene was used to normalize for differences in input cDNA. Pre-developed TaqMan Assays were used (Applied Biosystems, Foster City, CA, USA) and reactions were run on an ABI 7500 (Roche Diagnostics, Almere, The Netherlands) according to manufacturers description. Each sample was run in triplicate and the expression ratios were calculated using the ΔΔCT method after prior validation of the method for each target.

Results

Genome wide gene expression profiling was performed for initial leukemia and corresponding relapse samples (N=46) of 23 pediatric AML patients. The majority (19 out of 23 patients, 79%) of the patient group was male, the median age at presentation was 13.2 years and patients belonged to standard, intermediate and poor cytogenetic risk groups. Seventeen patients (74%) died after relapse. Overall patient characteristics are summarized in Table 1 and described more in detail in Supplementary Table 1 (Appendix).

We screened for mutations in a selected panel of genes (*NPM1*, *CEPBA*, *FLT3*, *NRAS*, *KRAS*, *PTPN11*, *KIT* and *WT1*) that were previously shown to associate with outcome in AML[11,21] in both the initial and corresponding relapse AML samples; results are shown in Supplementary Table 1 (Appendix). Analogous to our previous observations[21] both gains and losses of mutations between initial and relapse AML samples occurred in 8 out of 23 (35%) patients. In 10 out of 23 patients cytogenetic analysis was performed on both initial diagnosis and relapse samples. Although the major chromosomal rearrangements we detected in all paired samples remained stable, we observed additional cytogenetic aberrations in 7 out of these 10 paired samples (Supplementary Table 1, Appendix).

Comparison of initial with relapse expression profiles

To evaluate how well the initial samples of individual patients resembled the corresponding relapse sample, unsupervised hierarchically clustering was performed using a ‘one minus’ centered correlation and average linkage. For this analysis GEP data

of all 46 patient samples were preprocessed and probe sets were selected with a 1.5 fold variation in at least 2 samples. Figure 1 depicts how samples cluster together. The correlation between the initial and relapse samples with a patient varied between rs=0.15 and rs=0.75 (as illustrated by the difference in length of the branches in Figure 1). In 11 out of 23 patients (48%) the GEP of the initial sample did not cluster together with the GEP of the relapse sample. In 9 out of these 11 patients, the GEP of the initial sample was observed to cluster more than one branch away from the corresponding relapse GEP, indicating dissimilar GEP between the two disease stages for these individual patients. Remarkably, 5 out of these 9 paired samples (55%) also showed a shift in mutational status between the initial leukemia and relapse sample (Figure 1).

Table 1. Clinical characteristics of the 23 childhood AML patients in this study at presentation and first relapse

Pair	Sex	FAB type	Cytogenetics	Blast % diagnosis	Blast % relapse	Time to relapse	Follow up time	Dead
1	M	M5	MLL	90	86	9.7	10.1	Yes
2	M	M2	Normal	93	82	3.7	9.2	Yes
3	M	M2	Other	86	90	10.4	18	Yes
4	M	M4	t(6;9)	89	88	8.5	14.8	Yes
5	M	M5	Complex	88	84	8.9	9.2	Yes
6	M	M1	Unknown	91	86	14.8	19	Yes
7	M	M5	t(6;9)	93	89	5.7	12.3	Yes
8	M	M5	Other	96	82	15.8	26.3	Yes
9	F	M5	MLL	99	90	6.2	NA	No
10	M	M1	Unknown	82	93	11.4	14.9	Yes
11	M	M2	AML-ETO	91	94	58.4	164.7	No
12	M	M2	AML-ETO	91	91	12.6	101.2	No
13	M	M2	AML-ETO	88	83	8.9	15.5	Yes
14	M	M0	Complex	93	91	2.6	20.8	No
15	M	M2	AML-ETO	95	97	7.7	18.5	Yes
16	F	M4	Other	90	89	9.8	19	Yes
17	M	M4	MLL	93	83	5.9	9.1	No
18	F	M2	Normal	84	81	15.3	16.3	Yes
19	M	M2	AML-ETO	95	93	14.1	53.7	No
20	M	M4	Other	89	96	8.7	20.6	No
21	M	M4	Normal	86	88	6.9	9.9	Yes
22	F	M4	MLL	90	95	8.7	21.5	Yes
23	M	M5	Del 7	93	92	14.4	215.7	Yes

Time to relapse and follow-up time are given in months; Blast % after enrichment

This means that still 4 out of the 9 patients showed a lack of correlation between initial leukemia and relapse GEP, despite a stable mutational status in the commonly affected genes that were determined in this study. For the other 12 patients, initial and relapse

samples clustered together, indicating similar GEP at both disease stages. In 3 out these 12 patients (25%) mutational shifts of the analyzed genes were observed. Interestingly, patients with a GEP shift at relapse had a shorter TTR (median 8.7 vs 10.4 months) and a significantly worse OS (log-rank: P=0.04, Hazard ratio: 2.8, P=0.05).

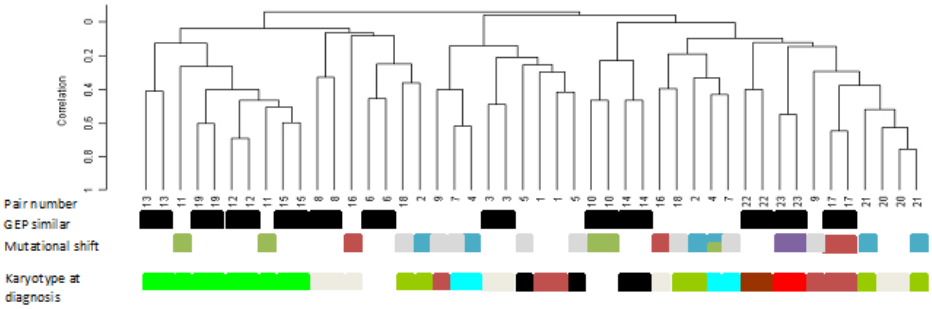

Figure 1. Hierarchical clustering dendrogram of paired initial and relapse AML samples. Similarity of GEP: Black bars indicate paired samples with GEP that correlate according to hierarchical cluster analysis. Grey bars indicate paired samples that lack both correlation in hierarchical cluster analysis and mutational shifts between initial and relapse AML samples. Mutational shifts: Blue, green, red and purple bars represent FLT3/ITD, WT1, RAS and PTPN11 mutational shifts respectively. Cytogenetics at initial diagnosis: dark red, bright red, blue, black, dark green, bright green, grey and white space represent MLL, monosomy 7, t(6;9), complex, AML-ETO, normal, other and unknown karyotype groups respectively.

Differentially expressed genes in initial and relapse pediatric AML samples

To first assess differential expression of individual genes that discriminate between initial and relapse samples in general, irrespective of patient specific characteristics, we compared GEP of the 23 initial with the 23 relapse AML samples (comparing the class of diagnosis to the class of relapse expression profiles). Even with a low stringency false discovery rate of 0.3, SAM analysis yielded only few individual genes that were discriminative between initial and relapse samples (Supplementary Figure 1, Appendix). Stratification according to cytogenetic subgroups did not improve these results (data not shown). With the aim to find relapse specific gene expression signature rather than individual genes that discriminate relapse from initial samples, we developed a multi-gene classifier using BRB Arraytools. The classifier consisted of 306 probes (228 known genes) that were discriminative between initial and relapse AML samples with a high sensitivity (70-83% depending on the cross validation method applied, $P_{306th\ probe}$ = 0.005, Supplementary Table 2 Appendix, Figure 2). Hence, there seems to be a set of genes that defines relapsed AML. Since AML patients are genetically heterogeneous,[8,32] we hypothesized that relevant differences are better studied by focusing on individual patients and their patient specific differences between diagnosis and relapse. To this end, we individually compared gene expression profiles of the initial samples with the corresponding relapse sample of each patient (n=23). This allows taking patient specific differences between diagnosis and relapse samples into account. Differential gene expression was determined for each individual patient by selecting genes that minimally had a two-fold change in expression level between diagnosis and relapse (the 23 lists of differentially expressed genes are shown in Supplementary Table 3, Appendix). In all 23 patients, more genes were up-regulated (median 422, range 40-1743 genes) than down-regulated (median 277, range 53-1326 genes) at relapse. Ingenuity pathway analysis was performed for these differentially expressed genes for each patient. The three most commonly affected pathways involved

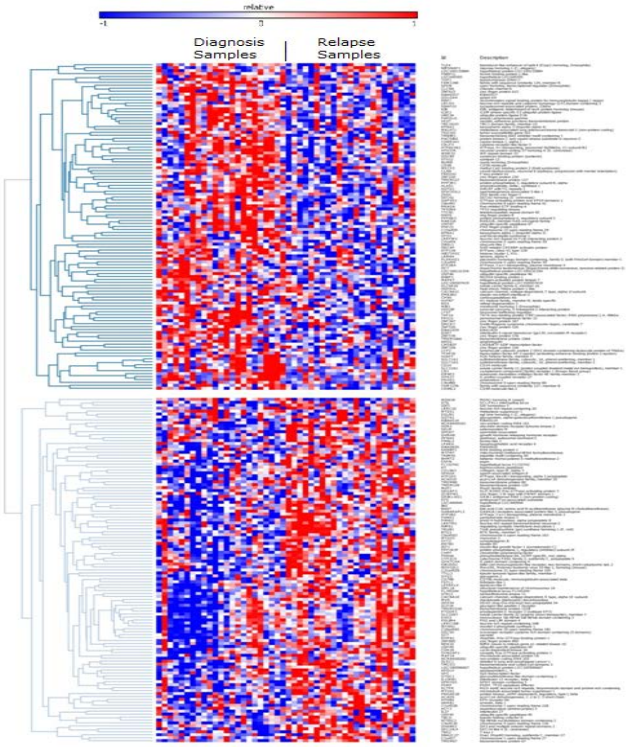

Figure 2: Heat map of probe-sets that distinguish initial from relapse AML samples.

inflammatory disease related networks (21/23 patients), cell movement and proliferation networks (15/23 patients) and chromatin disorder networks (13/23 patients).

In addition, Inqenuity pathway analysis for each individual patient predicted which transcription factors could be responsible for the observed gene expression patterns based on experimentally observed relationships between transcription factors and gene expression (Supplementary Table 4, Appendix). Table 2 shows the top 5 transcription factors that were predicted with high significance to be responsible for the differential gene expression between initial and relapsed samples. Some transcription factor associated pathways significantly predicted differential gene expression in the majority of patients.

In particular, *CEBPA, GFI1* and *SATB1* that were affected in 20, 16 and 15 out of 23 patients, respectively. Either one of these transcription factors was predicted to be involved in the gene expression profiles of all 23 patients. Network visualization plots show which transcription factors were involved and their target molecules that were at least 2 fold differentially expressed for individual patients (Supplementary Figure 2, Appendix). For example, in patient 3, differential gene expression of the target molecules was predicted to result primarily from *CEBPA, GFI1*, *SATB1* and *TBP1* activation/ inhibition (Figure 3). In addition, other transcription factors, such as *CEBPD*, *BCRA1*, *MYC*, SRF, and *TAF4B* were also significantly involved in this patient. The above described results implicate molecules and pathways that are involved in epigenetics. In concordance with these observations, differential expression of histone variant genes was observed in all analyses (Supplementary Figure 1, Appendix) and in the differential gene expression profiles of individual patients.

Table 2 Top list of transcription factors that regulate differentially expressed genes in paired diagnosis and relapsed samples, ranked by incidence

Transcription Factor	Median *P* value (range)	Percentage of patients	Top target molecules in data set (portion of patients)
CEBPA	2.6 $\times 10^{-5}$ (2.0×10^{-03}-1.5×10^{-09})	87	MPO(12/20), S100A9(12/20), ID2 (11/20), SOD2 (11/20), ANXA1 (11/20), CXCR4 (10/20), BTG1 (10/20), H1FX (10/20)
GFI1	8.2 $\times 10^{-03}$ (4.4×10^{-02}-1.1×10^{-05})	69	IL8 (14/16), ELANE(10/16), AZU1(9/16), RB1 (8/16), CDKN1A (6/16), SERPINA1(6/16)
SATB1	4.33 $\times 10^{-03}$ (2.7×10^{-03}-4.6×10^{-05})	69	HBB (12/16), HSPAA90 (11/16), RGS1 (11/16), CLEC2B (8/16)
KLF2	7.1 $\times 10^{-03}$ (2.3×10^{-02}-1.2×10^{-05})	65	IL8 (13/15), CCL3(12/15), CCL4(8/15),IL1B(7/15),PTGS2(7/15),SELL(7/15)
TBP	3.1 $\times 10^{-03}$ (4.7×10^{-02}-5.9×10^{-05})	61	TNFAIP3 (14/14), NFKBIA (10/14), IER3 (9/14), BCL2 (5/14), HLA-A (5/14)

In addition, regression analysis showed that the expression of individual histones variants correlated strongly with the expression of other cell cycle independent histone variants (Supplementary Table 2, Appendix). To confirm these findings, we validated the differential expression of *HIST1H1C* and *HIST1H2BG* by Taqman RT-PCR in an independent set of paired initial and relapse AML samples (n=8) (Supplementary Figure 1, Appendix). These results confirm the down regulation of histone gene expression levels at relapse and thereby indicate the importance of epigenetic features in relapse development.

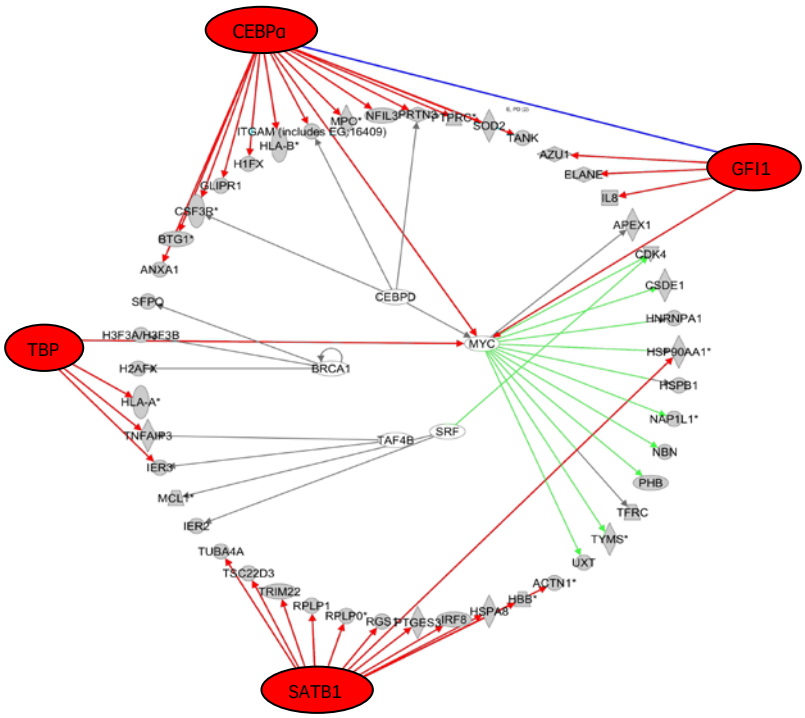

Figure 3 Transcription network plot for patient 3. The plot shows transcription factors (outer ring/ inner ring) that are predicted responsible for differential expression of shown target molecules (middle ring) between diagnosis and relapse of patient 3. A few transcription factors (CEBPA, GFI1, SATB1 and TBP) are responsible for the major changes in the differentially expressed target molecules.

Discussion

Improvements in OS for AML are likely to come from personalized targeted treatment approaches that aim to eradicate persistent leukemia. With the aim to elucidate factors that characterize pediatric relapsed AML, we performed a genome wide gene expression study on both initial and relapsed AML samples from 23 patients.

In 11 out of 23 (~48%) initial and relapsed AML samples the gene expression profiles did not end up in the same cluster (Figure 1) and hence differed significantly. Also in cases where the GEP of initial and relapse samples clustered together, differentially expressed genes could be identified. To elucidate biological factors that are relevant for relapse development, we determined which genes and thereby their underlying transcription factors, were changed between initial diagnosis and relapse within each patient. In the majority of patients (86%), *CEBPA* transcription factor related gene expression had changed at relapse. *CEBP* transcription factors play a crucial role in hematopoiesis;[33] it is indispensable for differentiation of myeloid progenitors along the granulocytic lineage.[34,35] Regulation of lineage-specific gene expression is known to occur via direct interaction with the basal transcriptional apparatus (*TBF/TFIIB*), but also via interaction with the *SWI/SNF* chromatin remodeling complex that modulates gene expression in an epigenetic manner.[36] Different types of *CEBPA* mutations are implicated in leukemogenesis[37,38] and confer a good prognosis subgroup in both adult and pediatric cytogenetically normal AML.[39,40] In our selected relapsed AML patient group, only one patient showed a single *CEBPA* mutation. It has also been shown that the normal function of *CEBPA* in hematopoietic differentiation can be altered for example via oncogenic lesions,[41] fusion proteins[42,43] or epigenetic alterations.[44] This may explain why in our study, genes that are regulated by *CEBPA* often showed an altered expression, while *CEBPA* expression itself was not significantly changed. For example, the cell surface glycoprotein CD9 is down regulated in bi-allelic mutated *CEBPA*[45] and its expression was also down regulated in our relapsed AML samples. Commonly, CD9 has a low expression on CD33 positive myeloid progenitors and plays a role in the regulation of cell differentiation, growth and motility[46]. Similarly, the expression of other molecules with a lower expression in more primitive cells, such as *CD14*, *CD58* or *CXCR4*, was lowered in relapse samples when compared to their individual initial AML samples (Supplementary Table 2, Appendix). These results indicate a *CEBPA* transcription factor related change in differentiation status of leukemic cell populations during disease progression. This may result in more primitive characteristics of the leukemic blast compartment at relapse when compared to presentation. Moreover, this implies a potential benefit for relapsed AML patients of therapy, that restores *CEBPA* related gene expression or normal differentiation, e.g. by gene therapy, nanoparticle based delivery of functional *CEBPA* or small molecule modulators of *CEBPA* or its downstream signaling.[47]

Another transcription factor of which the target gene expression levels were commonly (22 out of 23 patients) deregulated between initial and relapsed AML samples, was *SATB1*. This transcription factor is thought to be a key epigenetic modifier that links higher-order chromatin organization with gene regulation.[48] It regulates for example the expression of globin gene cluster,[49] many cytokines,[50] plays a role in hematopoietic differentiation[49,51–53] and is implicated in a variety of cancers and cancer progression.[54,55]

The observed down regulation of histone variant gene expression levels (e.g. *HIST1H1C)* also points at altered epigenetics in relapsed AML.[56] Histone variants may play a role in hematopoietic differentiation[57] and the expression of specific variants may be associated with leukemia.[58] Their functions are various, e.g. in the response to growth factors[59] or DNA damage response related apoptosis.[60] The expression of histone variants may be influenced by drug exposure[61] and may play a role in the tolerance to toxins.[62] These properties of histones have been exploited in the development on recombinant human

histone 1.3 (rhH1.3) as a cancer therapeutic agent, which was shown to induce apoptosis in leukemia cells by rupture of the plasma membrane. This drug was applied in a phase II trial for adult relapsed AML.[63]

The observed changes in gene expression from diagnosis to relapse and the patterns of clustering may be explained by the emergence at relapse of minor clones with different genetic make-up when compared to bulk of AML cells at diagnosis.[64] We have shown earlier that such minor clonal populations can be isolated retrospectively from diagnosis samples by FACS sorting. Besides a relapse specific mutational status, these cells expressed immunophenotypic markers that were specific for the bulk of leukemic cells at relapse.[65] Our current study is limited in the number of genes in which the presence of mutations was tested. It may be useful to assess relapse specific mutations and relapse specific gene expression and the link between them by next generation RNA sequencing in minimal residual disease cells that are likely to harbor the cells that initiate relapse.

In conclusion, we show a variable differential gene expression between initial and relapsed AML sample of individual patients. One group of patients shows a tumor evolution by which similarity between initial diagnosis and relapse gene expression profiles is lost (11 out 23 patients). The remainder of patients showed a more similar initial diagnosis and relapse gene expression profile. Our data show that relapsed AML has specific gene expression profiles that discriminate it from initial AML. The multiple pathways that were affected in individual patients may result from an epigenetic deregulation as suggested by observed the *CEBP* and *SATB1* transcription factor related differential gene expression and the diminished expression of e.g. histone variants at relapse. The biological characteristics of relapsed AML may be exploited in the application and development of novel strategies to prevent relapse or improve salvage therapies.

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