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Acute myeloid leukemia - Apoptotic signalling and gene expression associated with treatment response

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ACUTE MYELOID LEUKEMIA – APOPTOTIC SIGNALLING AND GENE EXPRESSION ASSOCIATED WITH TREATMENT RESPONSE

Marita Lagergren Lindberg

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“In order to get a big answer you need to ask a big question”

Peter Medawar, 1960

and this is just a small crack to let a little light in

Marita Lagergren Lindberg

To my family

In memory of my father

ABSTRACT

Acute myeloid leukemia (AML) is a severe, life threatening malignancy characterized by a clonal expansion of immature myeloid cells in the bone marrow, resulting in severe infections and bleedings. High dose chemotherapy is able to normalize the blood and bone marrow morphology (complete remission, CR) in a majority of treated patients, but recurrent disease, typically occurs within 1-2 years. Since further intensification of chemotherapeutic regimens is usually ineffective and accompanied by excess toxicity, novel approaches using better-targeted drugs are now being assessed. We have analysed the effects of one such new agent, gemtuzumab ozogamicin (GO) on AML cells and have also looked for biomarkers of clinical response and the role of multidrug resistance (MDR) expression utilizing biobanked cells from an AML cohort with known long-term therapuetic outcome. In **paper I** we analysed apoptotic signalling in response to GO, a monoclonal CD33 antibody conjugated to the DNA-double strand break-inducing toxin calicheamicin. The CD33 antigen is typically expressed on AML blast cells, but not on e.g. normal gut cells. We found that GO could induce mitochondrial depolarisation, activation of caspase-3 and decreased viability of primary cells from AML patients and AML cell lines. Moreover, we showed that GO activated the proapoptotic proteins Bak and Bax, regulators of mitochondria-mediated apoptotic signalling. Importantly, none of the above events could be observed in GO-resistant AML cells. In **paper II**, we looked at the role of caspase-2 in GO- or daunorubicin-induced apoptotic signalling. We noted that both drugs caused cleavage of caspase-2 into its active form. A selective caspase-2 inhibitor prevented GO-induced caspase-3 activation, yet did not influence the activation of Bak and Bax. All in all, our data indicate that both mitochondria-dependent and independent routes to caspase-3 activation are involved in GO-induced apoptotic signalling, findings that may lead to novel future therapeutic approaches for AML. Improved predictive biomarkers for treatment response are clearly needed to enable more personalized and effective therapeutic options in AML. In **paper III** we studied peripheral blood cells from 42 patients diagnosed with AML and subjected to induction chemotherapy, aiming to identify biomarkers of CR duration using global gene expression analysis (Affymetrix®). Prominent differences in gene expression were found with a remarkable up-regulation of the transcription factor RUNX1T1 in patients with short vs. those with long subsequent CR duration. Network analyses (Oncomine®) revealed multiple transcription factors as interactors to RUNX1T1, out of which TCF3 was also significantly up-regulated in patients with short CR duration. An *in silico* validation, taking advantage of previously published data from two other independent AML cohorts revealed 52 genes to be regulated in all three cohorts. Among these genes CXCL3, ZMIZ1 and PRDX2 attracted a special interest due to their reported involvement in cancer, leukemia, apoptosis and proliferation. Thus, CXCL3 and ZMIZ1, with known involvement in tumorgenesis, had increased expression in poor responders whereas PRDX2, a tumour suppressor gene, instead showed a decreased expression. In **paper IV** we investigated the clinical relevance of 380 genes, reported to have a role in multidrug resistance (MDR) and analyzed 11 paired sampled from AML patients, collected at diagnosis and at time of relapse. Unsupervised hierarchical clustering showed that half of the cases had a similar expression pattern at both time points, whereas in the remaining patients the MDR genes became altered, suggesting clonal evolution. Patient-by-patient analyses showed signs of unique individual patient gene signatures and in 10 out of 11 patients an increase of at least one ABC transporter was observed at relapse. These findings call for a more broad signalling analysis of diagnostic and relapse AML blasts in order to improve chemotherapy response and thereby overall survival of the individual AML patient.

LIST OF PUBLICATIONS

I. Petra Haag, Kristina Viktorsson, **Marita Lagergren Lindberg**, Lena Kanter, Rolf Lewensohn, and Leif Stenke. Deficient activation of Bak and Bax confers resistance to gemtuzumab ozogamicin-induced apoptotic cell death in AML. *Experimental Hematology, 2009, June;37(6):755-66*

II. Petra Haag, **Marita Lagergren Lindberg**, Dali Zong, Lena Kanter, Magnus Olsson, Boris Zhivotovsky, Bo Stenerlöw, Rolf Lewensohn, Leif Stenke and Kristina Viktorsson. Caspase-2 plays a role in mitochondria-independent apoptotic signaling in response to gemtuzumab ozogamicin and daunorubicin in acute myeloid leukemia. *Manuscript, 2013*

III. **Marita Lagergren Lindberg**, Petra Haag, Ali Moshfegh, Lena Kanter, Magnus Björkholm, Rolf Lewensohn, Kristina Viktorsson and Leif Stenke. Gene expression analyses at time of diagnosis indicate biomarkers predictive of therapeutic response in acute myeloid leukemia. *Manuscript, 2013*

IV. Chirayu Patel, Leif Stenke, Sudhir Varma, **Marita Lagergren Lindberg**, Magnus Björkholm, Jan Sjöberg, Kristina Viktorsson, Rolf Lewensohn, Ola Landgren, Michael M Gottesman, Jean-Pierre Gillet. Multidrug Resistance in Relapsed Acute Myeloid Leukemia: Evidence of Biological Heterogeneity. *Cancer. 2013 May 14. doi: 10.1002/cncr.28098.*

Additional paper

Jenny Forshed, Maria Pernemalm, Chuen Seng Tan, **Marita Lindberg**, Lena Kanter, Yudi Pawitan, Rolf Lewensohn, Leif Stenke, Janne Lehtiö. Proteomic Data Analysis Workflow for Discovery of Candidate Biomarker Peaks Predictive of Clinical Outcome for Patients with Acute Myeloid Leukemia. *Journal of Proteome Research, 2008 June;7(6):2332-41.*

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette transporter
ALL	Acute lymphoblastic leukemmia
AML	Acute myeloid leukemia
ANXA1	Annexin 1
APL	Acute promyelocytic leukemia
ATRA	All-trans-retinoic acid
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
CD33	Cluster of differentiation molecule 33
cDNA	complementary DNA
CEBPalpha	CCAT/enhancer binding protein alpha
CR	Complete remission
cRNA	complementary RNA
CXCL3	Chemokine (C-X-C motif) ligand 3
DAPI	4′-6′-diamidino-2-phenyllindole
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
Dsbs	Double strand breaks
EVI1	MDS1 and EVI1 complex locus
FAB	French-American-British classification
FDR	False discovery rate
FITC	Fluorescein Isothiocyanate
FLT3	FMS-like tyrosine kinase 3
FTI	Farnesyltransferase inhibitor tipifarnib
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gemtuzumab ozogamicin
GRO	Growth-related oncogen
GSR	Glutathione reductase
IPA	Ingenuity Pathway Analysis
LSS	the Life Span Study

MAPK	Mitogen-activated protein kinases
MDR	Multidrug resistance
MDS RAEB	Myelodysplastic syndrome refractory anemia with excess blast
MLL	Mixed-lineage leukemia
MNAT1	CDK-activating kinase assembly factor MAT1
MRD	Minimal residual disease
mRNA	messenger RNA
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NCBI	National Center for Biotechnology Information
NOLA2	H/ACA snoRNPs (small nucleolar ribonucleoproteins) gene family
NPM1	Nucleophosmin 1
OS	Overall survival
PBS	Phosphate buffer saline
PFGE	Pulse fied gel electrophoresis
POLH	DNAA polymerase eta
PRDX2	Peroxiredoxin 2
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative polymerase chain reaction
RUNX1	Runt-related transcription factor 1
RUNX1T1	Runt-related transcription factor 1; translocated to 1 (cyclin D-related)
SCT	Stem cell transplantation
SWOG	Southwest oncology group
tBid	Truncated Bid
TCF3	Transcription factor 3
TLDA	TaqMan Low Density Array
TMRE	tetramethylrhodamine ethyl ester
WBC	White blood cells
WHO	World Health Organization
ZMIZ1	Zinc finger, MIZ-type containing 1

1. ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a malignant disease characterized by an accumulation of immature myeloid blast cells in the bone marrow and most often in the peripheral blood. AML can also be present in other tissues such as in the skin, (leukemia cutis) [1] [2]. The clonal expansion of myeloid precursor cells in AML interfere with normal myelopoiesis and results in deficient function of normal blood cells which in turn leads to AML associated symptoms i.e. fatigue, bleedings and severe infections, some which are lethal. Immunophenotypic analysis by flow cytometry is a useful tool in AML in order to e.g. detect "myeloid" or "lymphoid" cell markers, making it possible to distinguish between minimally differentiated AML and acute lymphoblastic leukemia (ALL) [3] [4]. In mixed phenotype acute leukemia the blast populations express antigens characteristic of both myeloid and lymphoid lineages, which again makes flow cytometry analysis valuable [3]. Minimal residual disease (MRD) is defined as remaining leukemic cells in patients in whom morphological complete remission of the bone marrow has been achieved (described further in 1.1.2). Flow cytometry can also be used to detect MRD, since the technique can identify aberrant, malignant antigen combinations making it possible to distinguish leukemic cells from normal hematopoietic cells, with high sensitivity [5] [6]. AML is a severe life threatening disease. It occurs in all ages but is more common in the elderly [7] [8] with a median age at diagnosis of approximately 70 years [8]. Approximately 320 adults are diagnosed with AML in Sweden every year [9] making it the most common acute leukemia diagnosis in adults [10]. This corresponds to an annual incidence of 3-4/100 000 individuals, an incidence which is similar to that in other western countries [11] [9] [10].

1.1 BACKGROUND

In 1845 Rudolf Virchow (1821-1902) described patients at autopsy, with specific findings including splenomegaly and altered colour and consistency of the blood [12] [13]. Virchow proposed the term "leukemia", a greek word meaning "white blood" [12]. In 1891 new methods for staining blood cells were introduced, confirming that the myeloid leukemia cells were predominantly of granulocytic morphology [13].

Although "acute leukemia" has been a recognized disease since many years, it was not until the 1970s that a group of French, American and British leukemia experts (FAB) divided this entity into several subtypes. This classification was mainly based on the morphology, including the maturation stage of the dominating cell types of each subgroup. This FAB classification was introduced in 1976 [14] and modified in 1985 [15](Table 1A). Some leukemia subtypes in the classification system are linked to rather distinct symptoms, e.g. in patients with the M3 subtype (acute promyelocytic leukemia, APL), bleeding disturbances such as disseminated intravascular coagulation (DIC) are more frequently seen. The leukemic cells of patients with M0 to M5 are morphologically identified as precursors of white blood cells. FAB M6 and M7 are linked to immature forms of red blood cells and platelets, respectively. In order to incorporate the growing amount of knowledge, the World Health Organization (WHO) has introduced a novel classification system that takes into account the interrelation between morphology, cytogenetics, molecular genetics and immunologic markers, [3]

[16] (Table 1B). The aim is to make this system universally applicable and prognostically valid.

Table 1 Acute myeloid leukemia classification systems. (A) The French-American-British classification, dividing AML subtypes according to morphological characteristics of the leukemic cells. (B) The World Health Organization classification, also highlighting other features such as cytogenetic and molecular abnormalities.

(A)

FAB subtype	Name
M0	Undifferentiated acute myeloblastic Leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukeima with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4 eos	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

(B)

AML with recurrent genetic abnormalities - t(8;21)(q22;q22), inv(16)(p13;q22),t(16;16)(p13;q22) - t(15;17)(q22;q21), t(9;11)(p22;q23), t(6;9)(p23;q34) - inv(3)(q21;q26.2) eller t(3;3)(q21;q26.2) - t(1;22)(p13;q13) *Provisional entity: AML with mutated NPM1* *Provisional entity: AML with mutated CEBPA*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified - AML with minimal differentiation - AML without maturation - AML with maturation - Acute myelomonocytic leukemia - Acute monoblastic and monocytic leukemia - Acute erytroid leukemia - Acute megakarioblastic leukemia - Acute basofilic leukemia - Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

1.1.1 Etiology

The etiology of AML is generally unknown. It appears clear that previous treatment with chemotherapeutic agents [17], as well as preceding haematological malignancies, such as myeloproliferative disease and myelodysplastic syndromes, increase the risk of later developing AML. In some patients an association with exposure to ionizing radiation or benzene has been observed [18] [19]. Already in 1948 observant clinicians noticed a high incidence of leukemia in the population exposed to the atomic-bombs, which fell over Hiroshima and Nagasaki in August 1945, described by Folley and colleges [20]. This observation became an important starting point for the large epidemiological project, *the Life Span Study* (LSS), which has followed a sizable cohort of Japanese atomic-bomb survivors during several decades [18]. An increase in leukaemias occurred very soon after the blast (i.e. within the first 5 years), but the risk also subsided quickly and was dependent on the age of the exposed individual (i.e. a higher risk when exposed at a younger age) [18]. Benzene is regarded as a prototype for environmental leukemogenesis, where chronic exposure is associated with an increased risk of AML. It has been assumed, that AML derived from benzene exposure is similar to AML triggered by previous use of cytotoxic drugs to treat other malignancies [21],

although recent data have suggested a closer resemblance to *de novo* AML [19]. It is evident that previous treatment with chemotherapeutic agents [17], as well as preceding haematological malignancies such as myeloproliferative disease and myelodysplastic syndromes, increase the risk of later developing AML.

1.1.2 Treatment

In AML multimodal chemotherapy is used in order to re-establish normalization of the blood and bone marrow cell numbers and morphology, leading to normalization of the clinical status of the patient, a state defined as *complete remission* (CR) [22] [23] (Table 2).

Table 2: Complete remission criteria for response of treatment in AML patients according to the European LeukemiaNet [22] [23].

Complete remission
Bone marrow blasts < 5%; without the requirement of cell concentration in the marrow (counted ≥ 200 nucleated cells), with the absence of blasts with Auer rods, with presence of regenerating poeses
Abscece of extramedullary leukemia
Absolute neutrophil count > 1.0 x 10^9/L
Platelet count > 100 x 10^9/L
Independence of red cell transfusion[1]
No minimum duration of response required

[1] *This requirement is included in the international ELN guidelines, but has not been applied in the corresponding Swedish guidelines* [24] *, mainly because of varying policies for erythrocyte transfusions.*

Current standard induction chemotherapy treatment typically consists of an anthracyclin in combination with high-dose cytarabine [25] [26]. The first anthracyclin to be introduced was daunorubicin [27], which still, together with cytarabine, constitutes the cornerstone in modern AML treatment. Although a number of other antileukemic drug combinations have been introduced as induction treatment, none of them have been convincingly shown to be superior to daunorubicin/cytarabine (DA) [26] [17] [25]. In recent publications, a dose intensification of daunorubicin was suggested to be tolerable and resulting in superior survival [28]. A "full dose" induction treatment generally induces CR in approximately 70-80% of treated patients [29] [22]. In Sweden, almost all patients under the age of 70, half of the patients between 70 and 80 years and sporadic patients above 80 years of age, will receive intensive chemotherapy with curative intent [30] [31]. After achieving CR, a consolidation treatment, often with high doses of cytarabine (two to four courses), is generally given to prevent relapse of the disease [32] [33] [34]. One potential curative option for AML patients is allogeneic hematopoietic stem cell transplantation (SCT), usually performed

during first or subsequent CR. Despite intensive postremission therapy, and/or SCT, only a subgroup of patients with AML will be truly cured [35]. Even in the absence of morphologically detectable disease, at the time of transplantation, relapse post-SCT is a major cause of treatment failure [36]. Walter and colleagues have suggested that the minimal residual disease (MRD) status (i.e. the level of remaining disease after preceding chemotherapy), rather than the number of CRs, to be the most important factor to predict the risk of post-SCT relapse and long-term outcome [6] [37]. Most patients in CR will develop recurrent disease, often within 1-2 years. The long-term outcome in AML is therefore still poor, with a potential for long-term cure for approximately 40-45% of younger AML patients [38], and as low as <10% for patient cohorts above 60 years [39] [7] [28].

1.2 PROGNOSTIC FACTORS

Prognostic risk factors for AML (i.e. the risk of recurrent disease, usually linked to survival [39]) can be divided into 1) patient related factors 2) leukemia related factors and 3) response related factors which are further describes below.

1.2.1 Patient related factors

The most well established independent factor for poor prognosis is age [7] [40] [41] (Figure 1). The poorer outcome in elderly patients can be due to the fact that drug resistance and unfavourable cytogenetics are more commonly observed in this patient cohort [42] [43]. In addition, at older age the patients more often suffer from a poor performance status [44] or severe comorbidities, by themselves independent patient related risk factors [45] [46]. Despite this there are reports suggesting that some elderly will benefit from intensive chemotherapy [47]. Hypomethylating agents, such as azacitidine and decitabine, can sometimes induce long-term disease control without necessarily achieving CR and are some time used as an alternative to intensive chemotherapy [48] [49] [50] [51]. In addition, poor performance status, comorbidity and high white blood cells are shown to be risk factors for early death (i.e. induction related death) [7] [40] [41].

1.2.2 Leukemia related factors

Leukemia related factors include cytogenetic aberrations, associated hematopoietic disease, therapy-related disease and a high amount of white blood cells at diagnosis [42] [52]. As described above, the cytogenetics (chromosomal banding analysis) is considered very important for classification and clearly provides a powerful method to differentiate biologically and prognostically subgroups of AML [3] [53]. Cytogenetic aberrations are detected in approximately half of all adults diagnosed with AML [54] [55] and there are specific aberrations that are strong determinants of prognostic outcome and therapeutic response. The aberrant karyotypes distinguish three clinically important prognostic patient categories: those with favourable, intermediate and unfavourable cytogenetic, respectively. Thus, these analyses are important for stratification and to guide treatment approach [3] [53]. In addition, the number of specific information that can predict treatment outcome include not only cytogenetics, but also an increasing list of molecular features such as somatically acquired mutations of genes, i.e. FMS-like tyrosine kinase 3 (FLT3), nucleophosmin 1 (NPM1), Mixed-

lineage leukemia (MLL), Wilms tumor 1 (WT1), CCAT/enhancer binding protein alpha (CEBPalpha) and MDS1 and EVI1 complex locus (EVI1) [56-58] [38, 59].

1.2.3 Response related factors

After start of treatment, there are several important signs that have been linked to long-term clinical outcome. One of these is the early assessment of bone marrow blast content after administration of induction chemotherapy, where the threshold value is set to 10% blast cells at day 15 from the therapy initiation [60] [61]. This value is prognostically relevant, since the degree of blasts that is cleared from the bone marrow may reflect chemotherapy resistance [62]. A well known parameter that reflects the risk of relapse and overall survival is the response to induction treatment, i.e. whether the patient requires one or several induction regimens to reach CR [63]. In addition, high MRD levels are associated with an increased relapse rate and inferior overall survival [64] [65] [66]. Measuring the presence of MRD throughout therapy, usually through flow cytometry, provides a possibility to make a more tailored treatment approaches [53].

As can be seen in the classification systems AML is not a homogeneous disease, but rather a group of diseases (Table 1). Decades of research have demonstrated that patients with AML differ widely both clinically (i.e. in response to standard treatment) and in molecular, genetic and epigenetic characteristics [50] [67]. The enormous heterogeneity in the latter appears to indicate that optimal management of AML will eventually involve many specific regimens, with APL being an obvious example. The identification of APL is important, since this AML subtype responds very well to drug regimens containing *all-trans* retinoid acid (a vitamin A analogue) and since bleeding complications are more common, but manageable. The prognosis for APL has changed from the worst of the AML subtypes to, currently the best [68].

1.3 PREDICTION – NEW STRATEGIES

To predict therapeutic response is becoming increasingly valuable in clinical management of AML patients. As described in solid tumours treated with targeted therapy, such as cetuximab in colorectal cancer [69], trastuzumab in metastatic breast cancer [70] and imatinib in gastrointestinal stromal tumours [71], reliable biomarkers to predict treatment response are necessary to select the optimal treatment for each patient. The heterogeneity in AML is reflected by a number of biological and clinical features that are used to predict the likelihood of response to a certain therapy. By dividing patients into different subgroups, a better survival prediction is allowed, but this has still limited impact on treatment strategies with a few exceptions (e.g. all-trans retinoic acid in APL) [72].

1.4 MULTIDRUG RESISTANCE

Despite intensive treatment in AML, usually consisting of DA (see 1.1.2), the long-term outcome is poor. Although advances in knowledge and understanding of the pathophysiology of AML have increased during the last decades, there have been only minor improvements regarding therapy. Resistance to chemotherapy is still a major obstacle. Cells selected for resistance to a single drug, might also show cross-resistance

to other structurally and mechanistically unrelated drugs, a phenomenon known as multidrug resistance (MDR) [73] [74]. MDR is mediated by families of genes encoding efflux transporters (both ATP and non-ATP dependent transporters), drug uptake transporters, DNA repair proteins and phase I and II drug-metabolizing enzymes and inhibition of different cell death pathways e.g. apoptosis [73] [74].

The ATP-binding cassette (ABC) transporters are important mediators of multidrug resistance in patients with cancer [75] [73]. This family now consists of 49 different types, subdivided into seven different categories (ABCA though ABCG), and are expressed in both normal and malignant cells [76]. Three of those groups contains ABC transporters involved in multidrug resistance [76]. A number of these ABC transporters are known to transport drugs commonly used in the treatment of AML, such as anthracyclines and vinca alkaloids [73]. ABCB1 (ATP-binding cassette, subfamily B, member 1; also known as MDR1/P-glycoprotein) is expressed in lymphocytes and to a high extent in hematopoietic stem cells [77]. Moreover, ABCB1 is highly expressed in leukemic blasts [78] and is the most extensively studied transporter found to be higher expressed in secondary leukemias and reported to be associated with poor prognosis [78] [74].

1.5 TARGETED THERAPY IN AML

AML remains a very aggressive cancer disease with severe prognosis. Increased survival among younger AML patients, with further intensification of chemotherapy, is limited by toxicity and compromised by reduced compliance [79] [80]. One possible strategy to circumvent the toxic side effects is to use a treatment, which is directed specifically against the leukemia cells in combination with standard chemotherapy. Much effort has been invested during the last few decades in identifying molecular and genetic aberrations in AML. Although leukemogenic mutations such as those in *CEBPA, FLT3*, and *NPM1,* have been identified [56] [59] [38], recurrent genetic lesions appears to be insufficient in explaining the biological diversity of clinical AML. Epigenetic changes in chromatin structure, such as histone acetylation and methylation status are now intensively studied and are becoming important for the development of personalized therapy [81] [82] [83]. During the last few years there has been considerable research on targeted drugs, including small molecules and antibodies. For the treatment of AML, one promising antibody is gemtuzumab ozogamicin (GO; Mylotarg®), which was studied in the current thesis.

1.5.1 Gemtuzumab ozogamicin

Gemtuzumab ozogamicin (GO; Mylotarg®) is a targeted therapy that consists of a humanized monoclonal antibody (immunoglobulin G4, (hP67.6)) directed against CD33, a cell surface antigen. The active component of GO, calicheamicin (N-acetyl γ_1 calicheamicin), is a highly potent antitumor antibiotic and a DNA-targeting toxin [84] [85] [86]. When GO binds to the CD33 antigen the complex internalizes into the cell lysosomal compartment. The linker between the antibody and the toxin is stable at physiologic pH but allows hydrolytic release of the calicheamicin moiety at low pH such as in the lysosomes [85]. The toxin is reduced to 1,4-dehyrobenzene enters the cell nucleus and intercalates within the minor grove of the DNA helix causes site-specific DNA double strand breaks (DNA dsbs) (Figure 1). The target antigen of GO, CD33, is

expressed on immature normal cells of myelomonocytic lineage in healthy bone marrow but is not expressed on hematopoietic stem cells or on endothelial cells in the gastrointestinal tract. More important for AML patient response, CD33 is expressed by the leukaemia blast cells in approximately 90% of all AML patients [87] [86]. The fact that CD33 is not expressed on mature hematopoietic cells or endothelial cells appears to provide a comparatively low GO-induced toxicity from healthy tissues [85] [88].

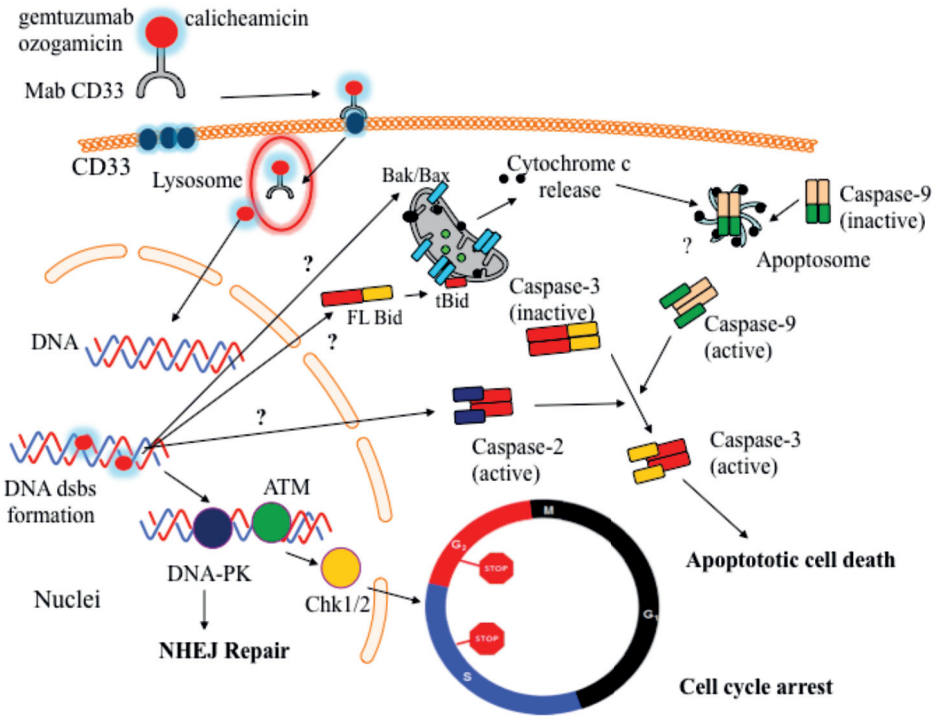

Figure 1: Gemtuzumab ozogamicin cellular signalling

GO was formally registered in the US for treatment of a subset of elderly AML-patients, i.e. patients who due to physical status were not considered fit to receive conventional high dose chemotherapeutics [89] [90] [91]. Post approval, additional clinical trials were prompted and the Southwest Oncology Group (SWOG) initiated S0106, a randomized trial, comparing GO in combination with standard induction therapy with daunorubicin (D) and cytarabine (A), versus DA alone. As a second randomization, the trial also tested whether GO given as a post-consolidation therapy could improve disease-free survival. The trial accrual was stopped early (in August 2009) when a higher early treatment-related mortality and no clinical benefit could be observed in the experimental group receiving GO, as compared to the comparator group receiving standard chemotherapy alone [91]. This led to a voluntary withdrawal of the drug from the US market. In Europe, GO has been used under license and in controlled clinical trials, with promising results [87]. Used as a single drug, GO has

shown clear anti-leukemic activity with clinically relevant responses in approximately 30% of AML-patients treated in relapse [90].

Recently, leading haematologists have made a case for reapproval of GO in AML based on results from four completed randomized studies supporting the efficacy of this agent in newly diagnosed AML with acceptable toxicity [92]. In acute promyelocytic leukemia (APL), GO is shown to be effective both as a single drug and in combination with *all-trans* retinoic acid (ATRA), likely because of high surface expression of CD33 in APL cases [93] [94]. A number of studies have indicated that GO improves survival also in subsets of non-APL patients, supporting that CD33 is a clinically relevant target in some AML patient subsets [95] [96] [97] [86].

1.5.2 Apoptotic signalling cascades

Apoptosis is a type of cell suicide program, which is found in all our cells [98]. Apoptosis is essential for clearance of the cells that are, in some way damaged, infected or at the end of their normal life span. In the body this type of death doesn't trigger an inflammatory response, as would be the case if necrotic cell death took place. The morphological hallmarks of apoptosis include DNA fragmentation, chromatin condensation, cell shrinkage and membrane blebbing [99] [100] [98]. Important for causing the hallmarks of apoptosis are caspases, a family of cysteine proteases [101]. Caspases can roughly be divided into either initiator (caspase-2, -8, -9 and -10) or executor (caspase-3, -6 and -7) caspases, the former being capable of autoproteolytical activation and trigger proteolytically cleavage of the latter, which carries out the selective substrate proteolysis giving rise to the apoptotic morphology features [102] [101]. In principal the apoptotic cell death signalling cascades can be triggered via an extrinsic or an intrinsic pathway [101], i.e. via death receptor or mitochondria-mediated route resulting in caspase activation. The death receptor pathway is exemplified by the binding of FAS ligand to the FAS receptor, leading to recruitment, dimerization and activation of caspase-8. Active caspase-8 will then activate the executioner caspases (caspase-3, -6 and -7) or activate the intrinsic pathway. The intrinsic pathway, also referred to as the mitochondrial pathway, is activated through different cellular stresses, e.g. growth factor deprivation, DNA damage among others [101], subsequently leading to activation of a subclass of Bcl-2-family of proteins, BH3-only proteins. Moreover, the BH3-only proteins play a critical role as they function to integrate signals from both DNA-damage as well as from growth factor receptors onto the Bcl-2 family proteins Bak and Bax, which are in part responsible for mitochondrial release of cytochrome c [103]. Bak, which is situated in the outer mitochondrial membrane, undergoes several N-terminal conformational changes [104] so that its multimerization and interaction with Bax is possible [105]. Bax, situated in the cytoplasm, respond to DNA damage by a conformational change that allows integration in the outer mitochondrial membrane and formation of homo- or hetero-complexes with Bak and promoting the release of cytochrome c [105]. The release of cytochrome c from mitochondria is critical for activation of the intrinsic pathway of apoptosis. This release leads to the formation of the apoptosome complex in which caspase-9 is cleaved, subsequently initiate the apoptotic cell demise by activation of caspase-3 giving rise to the nuclear apoptotic morphology [101] (Figure 1).

2. AIMS OF THESIS

The overall aim of this thesis was to identify and characterize prognostic biomarkers for clinical responses to anti-leukemic treatment in AML and to understand molecular signalling mechanism operative for the cellular response to gemtuzumab ozogamicin (GO), a targeted therapy of AML.

Specific aims

Paper I: To characterize GO-induced cellular and molecular events, linked to sensitivity and resistance in AML.

Paper II: To identify proteins of importance for GO-induced apoptotic signalling upstream of the mitochondria focusing on caspase-2, an apical known be involved in signalling associated to DNA damage-induced apoptotic signalling.

Paper III: To identify prognostic biomarkers at gene levels associated with clinical long-term response to chemotherapy in a defined AML patient cohort with known clinical outcome.

Paper IV: To identify resistance mechanisms involved in the acquisition of MDR in AML patients, using 380 genes chosen by their potential role in MDR reported over the past decades and by comparing their expression in AML patient samples collected at diagnosis and after recurrent disease.

3. PATIENTS AND METHODS

3.1 PATIENT COHORTS (paper I-IV)

In the present studies (*papers I-IV*), AML patient samples taken from biobanked material consecutively collected during 1987-2003 and stored at the Dept. of Hematology, Karolinska University Hospital Solna, Stockholm, Sweden, was used. The studies were approved by the central Ethics Review Board (KI 03-600 (*papers I-IV*) and 2007/1526-31/3(*paper II*), Karolinska Institutet and followed the declaration from Helsinki [106].

The samples consisted of peripheral blood collected from adult AML-patients at diagnosis, prior to treatment (*papers I-III*) and at the time of relapse (*paper IV*). Cells were isolated by Ficoll-Hypaque® separation and the mononuclear cells were freshly frozen and stored in the biobank. To gain knowledge of the patients' clinical condition and response to treatment approximately 200 medical records and individual patient data were collected and reviewed. Some samples were not of sufficient quality, and others re-diagnosed to be non-AML, limiting the amount of cases available. Patients with acute promyelocytic leukemia (APL) were excluded from these studies (*papers I-IV*) due to their specific treatment regimen. Those patients have a chromosomal translocation involving the retinoic acid receptor alpha (*RARα* or *RARA*) gene and are unique from other forms of AML in its responsiveness to *all-trans* retinoic acid (ATRA) therapy [107] [108].

Patient characteristics are shown in Table 3. In *paper I* six patients were analysed, five with AML and one with acute lymphoblastic leukemia (ALL). The patient with ALL was used as a negative control. In *paper II* we studied primary AML patient cells (n=22), for validation of results obtained from AML cell line *in vitro* analyses. In *paper III* the gene expression differences in AML patients with poor and good clinical outcome were compared. The patients in the training cohort (n=42) were stratified into two groups according to their complete remission (CR) duration (i.e. short CR: < 6 months and long CR: >6 months). In this training cohort the median CR duration was 161 days, which led us to set the cut-off level for shorter vs. longer CR duration at 6 months (n=24 and n=18, respectively). The two groups were perceived equal in terms of age, sex, white blood cell count, cytogenetic status and presence of preceding hematologic malignancy. In this study there were three patients whose diagnosis later was revised to ALL. For *in silico* comparison two independent cohorts were analysed and are described in section *3.3.2*.

In *paper IV* we analysed eleven paired samples (samples taken at diagnosis and after relapse) in a patient-to-patient analysis, to compare the gene expression between the two time points. Each patient sample here worked as their own control in finding gene expression differences after chemotherapy treatment and relapse as compared to the time at diagnosis thereby reducing the inter patient heterogeneity.

All patients in *paper III* (training cohort) and *paper IV* had an induction treatment with the intention to cure. The chemotherapy consisted of an anthracyclin in combination with cytarabine.

Table 3. Patient characteristics listed according to the analysis in the different papers

	Paper I n=6	**Paper II n=22**	**Paper III n=42**	**Paper IV n=11 (paired samples)**
Sex; female	3	15	27	8
Age (yrs)	61.5 (29-74)	67 (32-85)	62 (18-85)	58 (28-72)
WBC (x10^9/l)	59 (1.7-276)	56.8 (2.5-276)	40 (0.9-276)	32.2 (9-32.2)
Platelets (x10^9/l)	70 (24-167)	91 (17-303)	65 (8-303)	65 (33-303)
CR dur (days)	204.5 (79-304)	151.5 (12-3701)	161 (12-3701)	284.5 (48-1166)
OS (days)	480 (140-744)	303.5 (91-3772)	355 (91-3772)	563 (193-1664)
Cytogenetics				
Low risk	1	3	4	0
Intermediate risk	3 (3*)	11 (8*)	26 (20*)	7 (5*)
High risk	0	1	4	2
Unknown	2	7	8	2
FAB-classification				
M0	1	0	0	1
M1	1	11	13	3
M2	2	6	12	1
M4	1	1	5	3
M5A	0	1	3	1
M5B	0	3	5	2
M6	0	0	1	0
ALL	1	0	3	0

*Abbreviations; Age: age at diagnosis. WBC: white blood cell count at diagnosis. CR dur: complete remission duration. OS: overall survival. Age, WBC, Platelets, CR dur and OS are shown as median value (range). FAB-classification: French-American-British classification of acute leukemia. Intermediate risk (incl *normal cytogenetic profile).*

Cell lines (paper I-II)

The AML cell lines used for experiments in *paper I* were HL60, NB4 and KG1a, described earlier [109] [110] [111]. HL60 and NB4 cells are derived from human promyelocytic leukemia cells. KG1a is a cell line of immature myeloblasts, which was shown not to respond to colony stimulating factor or to anticancer drugs (e.g. daunorubicin and vincristine) [112] and also described by Amico et al., to be resistant to gemtuzumab ozogamicin (GO) [113]. In *paper II* HL60 cells were chosen as model system. For molecular studies the AML cells were seeded 24h or 48h prior to treatment and were in exponential growth when treated. Cells were incubated with the monoclonal antibody gemtuzumab ozogamicin (GO) (described in section 1.5.1) or calicheamicin at clinically relevant concentrations (10 to 1000 ng/ml and 0.3 to 30 ng/ml, respectively) for 24h to 72h. The concentration of calicheamicin used corresponds to the amount linked to the antibody (GO 100ng/ml contains 0.3ng/ml calicheamicin etc).

3.3 EXPERIMENTAL METHODS

3.3.1 Analyses of GO- induced molecular events (paper I-II)

Cell viability

The efficacy of GO, calicheamicin and etoposide exposure was characterized by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) cell viability assay after 24h, 48h or 72h of continuous drug treatment. The MTT assay measures the capacity of cells to reduce the MTT to unsoluble formazan, giving a purple colour. The formation of the formazon crystals is directly proportional to the number of living cells and was measured by spectrophotometer. In *paper I* absorbance was set to 100% in untreated cells and viability of the treated cells was determined accordingly. In *paper II* (which also includes daunorubicin), cell viability was analysed after using trypan blue stains. In this method non-viable cells will have a leaky membrane and therefore are stained blue whereas viable cells, i.e. cells with intact cell membranes are unstained. Cells were counted using phase contrast microscopy. Viability prior to treatment was set to 100%, to which treated cells were related.

Apoptosis assessment

In *paper I* and *II* induction of apoptosis after GO or calicheamicin treatment, were assessed by analysing nuclear apoptotic morphology. The cells were centrifuged onto slides, fixed and nuclei stained using mounting media containing 4´-6´-diamidino-2-phenyllindole (DAPI). DAPI is a dye that binds strongly to DNA at A-T rich regions. DAPI staining of normal cells will give a uniform staining, whereas in apoptotic cells fragmentation of DNA will give a punctate staining pattern. Images were acquired on fluorescence microscopy (ZEISS Axioplan 2 with a Zeiss x63 lens).

Mitochondrial dysfunction

To analyse the GO-induced effects on mitochondrial depolarization in *paper* I we measured the loss of mitochondrial membrane potential using tetramethylrhodamine

ethyl ester (TMRE). TMRE is a lipofilic dye, which ”gets stuck” in the mitochondria if the potential is present. If the cell is apoptotic, the mitochondrial membrane looses the potential and the dye leaks out of the cell. TMRE-associated fluorescence is measured in the Fl-2 channel in flow cytometry and is presented in histograms with peak shifting to the left if mitochondria membrane is leaking (i.e. low TMRE staining) (*paper I*).

Activation of the proapoptotic proteins Bak and Bax are central events in apoptosis signalling [114] and involves conformational changes in the N-terminus of each of them, followed by multimerization. We examined GO-induced activation of these proteins by using antibodies recognizing these activity related conformation changes of Bak and Bax respectively (*paper I-II*), followed by flow cytometry-based quantification. In both *paper* I and *paper II* activation of caspase-3 was analysed using an antibody that recognize the active site, DEVD, of caspase-3 (*paper I-II*) [98]. The antibody used is conjugated to Fluorescein Isothiocyanate (FITC) that allows detection by flow cytometry. Activated caspase-3 was quantified and presented as fold change mean value as compared to untreated cells, which were set to one.

Western blot analysis of protein expression

In *paper I* and *paper II* protein expression analysis were performed using western blot. Total cell extracts were made and proteins separated using SDS page gels. After transfer onto nitrocellulose membranes the membranes were probed with antibodies recognizing phosphorylated and total form of p38 MAPK (*paper I*) and full length and cleaved fragments of caspase-2, PARP-1, caspase-3 and Bid (*paper II*) with GAPDH as a loading control. Membranes were incubated with secondary goat-anti-mouse- or goat-anti-rabbit-antibodies and protein expression were detected using enhanced chemoluminescent + method (*papers I-II*), protein banding intensity quantified by Quantity One software. In *paper II* we also used the Odyssey® Sa Infrared Imaging System (LI-COR). Primary AML cells were analysed for expression of caspase-2 (n=17) and caspase-3 (n=19)(*paper II*). The loss of patients was due to methodological problems.

Identification of DNA double strand break formation

In *paper II* we analysed DNA double strand break (dsb) formation after treating HL60 cells with GO, calicheamicin or etoposide using pulsed field gel electrophoresis (PFGE). In PFGE, the DNA fragments are separated based on size and hence if DNA dsbs are present DNA fragments of lower size are detected. The protocol used was optimized for separations of DNA fragments in the size range 1-10 Mbp [115] and data in each treatment is presented as fold fragmented DNA (fragments <5.7 Mbp) relative to untreated cells.

The role of caspase-2 in apoptotic signalling

In *paper II* we assessed the effects of GO and daunorubicin in cell lines, and the effects of GO in primary cells from AML patients, focusing on caspase-2 as a part of the apoptotic induction signalling. In order to analyse if GO-induced caspase-3 and apoptotic signalling in AML cells were dependent of caspase-2, HL60 cells were pre-treated with the caspase-2 inhibitor z-VDVAD before chemotherapy treatment (*paper II*). z-VDVAD-fmk is a synthetic peptide that irreversibly binds and inhibits the active site of caspase-2 and thereby its function is blocked [116]. After pre-treatment with the

caspase-2 inhibitor cells were treated with GO, calicheamicin, daunorubicin or etoposide before analysing caspase-3, Bak/Bax activation and cleavage of full length Bid into tBid as outlined above.

Statistical analyses

Data presented were expressed as the mean values ± standard deviation (S.D.) of at least three independent experiments. Significance differences between untreated and treated samples were calculated using t-test. For comparison of caspase expression in primary AML cells two-tailed Mann-Whitney t-test was applied.

3.3.2 Gene array analyses: expression and validation (paper III)

Preparation of RNA and synthesis of cDNA

In *paper III* gene expression of AML patient derived cells were studied using Affymetrix U133 2.0 Plus GeneChip array (www.affymetrix.com). RNA was isolated from mononuclear cells obtained from 42 patients diagnosed with AML using RNA-Bee (Biosite, Stockholm, Sweden), which is a reagent for isolation of total RNA from samples of human origin. The RNA from each patient sample was pooled into two groups, short and long CR duration respectively and 500 ng of RNA from each group was subsequently transcribed into cDNA using reverse transcriptase. With cDNA as the template, *in vitro* transcription was made to synthesize biotin-labelled cRNA. The biotin-label is to ensure a high binding capacity to the chip, using streptavidin antibody. RNA was then fragmented in a fragmentation buffer.

Gene array analysis

For the gene expression profiling the fragmented cRNA (15 μg/probe array) was hybridized to the Affymetrix U133 2.0 Plus GeneChip array in a hybridization oven. The chips were washed and thereafter stained using streptavidin phycoerythrin conjugate (SAPE) followed by the addition of a biotinylated anti-streptavidin antibody and finally with streptavidin phycoerythrin conjugate. Probe arrays were scanned using a fluorometric scanner (Affymetrix Scanner) and the generated signal which depends on the strength of the hybridization determined by the number of paired bases will reflect the degree of mRNA expression. Gene array data chip were normalized using the gcRMA algorithm.

The gene expression data were analysed with GeneSpring G10X software and these analyses were made in duplicates. In this software, the unpaired t-test (threshold set to a p-value <0.05), "false discovery rate" (FDR) was set to $< 5\%$. For the experiment we compared the group with short CR duration to the group with long CR duration. Fold change was used for measuring changes in expression levels of each gene (mean value) when comparing the two groups. In the subsequent gene analysis we focused on genes showing at least a 2-fold (training cohort) difference in gene expression.

Real-time quantitative PCR

To validate the gene expression data we assessed the mRNA expression of some genes, pooled and individual samples, by real time quantitative polymerase chain reaction (RT-qPCR). In this method the mRNA sample is first reversed-transcribed to cDNA with reverse transcriptase and then amplified in the presence of a PCR reaction

mixture containing primers for the gene of interest, a DNA polymerase (usually Taq polymerase), deoxyribonucleotides (dNTP) and a fluorescent DNA-binding dye. To compare the expression of genes between different samples in *paper III*, the "housekeeping gene" GAPDH, which has almost a constant level of expression, was used for normalization of differences in mRNA content between analysed samples. Pooled samples were assessed for four genes shown to be up-regulated (RUNX1T1, TKTL1, U2AF1, NUDT4) and three genes found to be down-regulated (ANXA1, FLRT3, TLR8) when comparing patients with short CR duration to those with long CR duration. The expression of RUNX1T1 was also examined in individual patient samples in order to clarify that no single patient sample in the pooled material was an extreme "outlier" and thus responsible for the large difference in gene expression. Relative quantification was determined by the ΔΔCt formula. For statistical analysis of RUNX1T1 in individual patients the Mann Whitney test was applied.

Ingenuity Pathway Analyses

To interpret the biological meaning of the gene expression data in the AML samples analysed in *paper III*, the gene expression alterations were subjected to the web-based analysis tool Ingenuity Pathway Analysis® (IPA, www.ingenuity.com). IPA is a computerized tool that integrates omic data on basis of published prior knowledge created in the Ingenuity Knowledge Base. In *paper III* we specifically used IPA to create a custom network which was based on RUNX1T1 in order to find possible interaction partners and their regulation in our data set. In IPA, the top gene function was detected by Fisher´s Exact Test (threshold $p<0.05$).

In silico validation

In *paper III*, *in silico* validation of obtained gene expression results were made using publically accessible data sets with gene expression results and corresponding clinical data. Via oncomine (www.oncomine.org), published gene expression data was filtered for I) cancer form: *Leukemia*, II) clinical outcome: *Survival status*, III) platform: *Affymetrix*. Two AML cohorts met this selection, which were used for validation. For validation cohort 1 data from Meztzeler et al, which contained 162 AML patients and one with refractory anemia with excess of blasts (MDS RAEB), were used [117]. All patients had a normal karyotype and patient characteristics contained data on FAB classification, age, tissue and overall survival. Validation cohort 2 was obtained from Raponi et al, who investigated efficacy and safety of the farnesyltransferase inhibitor tipifarnib (FTI) in a single-arm phase-2 study of elderly AML patients (n=34) [118]. Data included age, gender, unfavourable karyotype and overall survival. Patient samples from both cohorts were collected before treatment and consisted of bone marrow cells, but also two samples from peripheral blood [117]). In both studies the gene array raw data had been uploaded in NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo), were it was accessible together with individual patient characteristics for our *in silico* validation.

The gene array raw data from analyses of validation cohorts were downloaded and analysed together with the raw data from the training cohort (our own gene expression data) using GeneSpring G10X software as described above. In the validation cohorts there were no information of CR duration of the patients but the overall survival (OS) was known. When analysing the training cohort, a clear association between short CR

duration and short OS, was seen as previously been described for AML patients [39]. Therefore OS was used as a parameter for prognosis in the validation cohorts where patients were divided in two groups according to OS. The cut off was set to the value of median overall survival in respective cohort. In validation cohort 1 cut off was set to 280 days (short OS n=83, long OS n=80) and in validation cohort 2 cut off was set to 235 days (short OS n=17 and long OS n=17). To ensure that the sample handling or differences in the analyses (i.e. preparation of the raw data) were not responsible for the observed differences in gene expression patterns, a comparison of gene expression was first made within each cohort. The results from each cohort were then compared to each other.

3.3.3 Gene expression of possible multidrug resistance mechanisms (paper IV)

Preparation of total RNA

For the gene expression studies of possible multidrug resistance mechanisms in AML (*paper IV*) peripheral blood samples from eleven AML patients were used, including samples collected both at diagnosis and at relapse. The total RNA was extracted with the Trizol. cDNA was synthesized from 1 μg of total RNA using High Capacity cDNA kit with RNase inhibitor. This kit uses random primers for reverse transcription of RNA into cDNA.

TaqMan Low Density Arrays

TaqMan Low Density Array (TLDA), which is a highly sensitive and specific TaqMan-based RT-qPCR [75], was applied. The choice of 380 genes for analyses of chemotherapy resistance was done based on their previously reported role in MDR [119]. The genes identified were confined to several different biological mechanisms including apoptosis, drug uptake or efflux, tumour transformation, tumour suppressor activity, stress response, DNA repair, signal transduction and phase I and II drug-metabolism [120]. The TLDA card, which is a 384-well custom-made array for the 380 MDR-associated genes, were thus used in which cDNA were mixed with the TaqMan Universal PCR Master mix, then loaded into reaction reservoirs and centrifugally spread into each reaction chamber.

Data analyses

Collected data from TLDA (i.e. the eleven paired AML samples) were analysed using *BRB ArrayTools* (http://linus.nci.nih.gov/BRB-ArrayTools.html), a method developed for visualization and statistical analyses of microarray gene expression data. To focus on genes that are more likely to be informative, genes were filtered out if they were expressed in less than 50% of the samples.

Statistical analysis (paper IV)

For correlation to FAB-classes and for the expression of each genes, Spearman rank test with the threshold $p < 0.05$ was applied and for correlation of gene expression with the CR duration, data were subjected to a Cox proportional Hazards test. False Discovery Rate (FDR) for each gene was calculated using the Benjamini-Hochberg method. Pairwise comparisons were manually performed using the ΔΔCt method [121].

4. RESULTS

Treatment with high dose chemotherapy often leads to severe side effects that sometimes are even lethal. At the same time, we aim at reaching a better long-term effect and need to kill remaining leukemic cells, in order to cure. With current available treatment options, this is only reached for a minority of the patients and most of them will have a recurrent disease within 1-2 years. In *papers I and II* we investigate the targeted therapy gemtuzumab ozogamicin (GO), where the precise cellular and molecular mechanisms behind the clinical response of GO treatment remain rather vague. However, a more specific knowledge of the mechanisms of cell death induced by GO could pave way for identification of possible molecular determinants for *in vivo* GO responsiveness and help to reveal AML patients, for whom GO may be of value as treatment. The high amount of relapse in AML is a large problem, why the need of improved prediction of chemotherapy response and knowledge of resistance mechanisms in AML, is necessary. Predictive biomarkers for response and response duration are required and mechanisms for therapy resistance need to be identified. These research issues were in focus in *paper III* and *paper IV,* respectively.

4.1 PAPER I

Deficient activation of Bak and Bax confers resistance to gemtuzumab ozogamicin-induced apoptotic cell death in AML

In *paper I,* we focused on proapoptotic mitochondrial mediated signalling induced by GO. Initially, cells were incubated with GO or free calicheamicin in clinically relevant doses (10 to 1000 ng/ml and 0.3 to 30 ng/ml, respectively) showing a clear dose and time dependent decrease in cell viability in GO-sensitive cell lines (HL60 and NB4), as compared to untreated cells. In contrast, this was not the case in the KG1a cell line, which is GO resistant, although KG1a cells responded with decreased cell viability when treated with etoposide, another DNA-damaging agent.

Since the active part of GO, calicheamicin, causes site-specific DNA dsbs [122], which may induce apoptosis through the intrinsic apoptotic pathway, we examined activation of the mitochondrial pathway after GO administration. Activation of mitochondria is an early apoptotic event in which cytochrome c is released and the apoptosome is formed, resulting in activation of caspase-9 and subsequently of caspase-3 (Figure 1) [114] [87]. We observed that GO induced depolarization of mitochondria and activation of caspase-3 in the sensitive HL60 cells, while no such event was observed in GO-resistant KG1a cells. Similar activation was revealed using free calicheamicin or etoposide. In contrast, GO-resistant KG1a cells did not reveal the activation of caspase-3 after GO-exposure. A 2-fold increase in active caspase-3 was however observed in KG1a cells when exposed to clinical relevant doses of either calicheamicin or etoposide, showing that activation of caspase-3 is functional in KG1a cells. The results suggests that GO and calicheamicin activate the intrinsic pathway for apoptosis in GO sensitive cells.

Conformational changes in Bak and Bax leading to their proapoptotic status are central in mitochondria-mediated apoptotic signalling and precede depolarization of mitochondria [114] [123]. Using antibodies recognizing the specific activity-related conformational changes in Bak and Bax, we examined the activation of those proteins

[124] [125]. Importantly, activation of Bak and Bax in response to GO was found in GO-sensitive HL60 cells increasing sixfold and fourfold respectively, when treated with GO 1000 ng/mL. Treatment with calicheamicin gave similar results regarding both Bak and Bax, whereas in GO-resistant KG1a cells neither GO nor calicheamicin were able to induce Bak/Bax activation.

DNA damage-induced apoptosis has been linked to a persistent activation of the stress-activated protein kinases (SAPKs) such as p38 MAPK [126]. We found that there was a dose-dependent increase in activation of p38 MAPK in GO-sensitive HL60 cells, with as much as a fourfold increase at the highest GO concentration whereas in GO-resistant KG1a cells no GO-induced activation of p38 MAPK was observed. Thus, our results suggest that activation of p38 MAPK is induced in GO-induced mediated cell kill.

We also analysed GO-response in primary AML cells and found that in CD33-positive blast cells derived from a treatment naïve AML patient, both GO and calicheamicin treatment resulted in approximately 30-50% reduced cell viability after 48h. In contrast, in CD33-positive cells from a heavily pre-treated AML patient in relapse, neither GO nor calicheamicin were capable of inducing any cytotoxicity. Moreover, cells from the ALL patient (in 2nd relapse) also failed to respond to GO treatment, but were inhibited by calicheamicin. In conclusion, our results from GO-treated primary AML cells and AML cell lines pinpoint the importance, not only of CD33 expression but also of p38 MAPK activation with subsequent initiation of mitochondrial depolarization and caspase-3 activation, as molecular determinants for clinical GO responsiveness in GO-sensitive cells.

4.2 PAPER II

Caspase-2 is a mediator of mitochondria-independent apoptotic signaling in response to gemtuzumab ozogamicin in acute myeloid leukemia

In *paper II* we aimed at understanding GO-induced apoptotic signalling up-stream of the mitochondria. As caspase-2 is known to play a role in DNA-damaging apoptotic signalling at this level [127], we focused our analysis on how it acts in GO-induced cell death signalling. By treating HL60 cells with GO (100 ng/mL and 1000 ng/mL) during 24h, using immunoblotting and GAPDH as a loading control, we demonstrated that GO triggered full-length caspase-2 cleavage to a 35 kDa cleaved fragment. Importantly, also in primary cells from three AML patients, treated with GO we showed that full length caspase-2 disappeared albeit no cleavage fragment was evident.

In *paper I* we showed that caspase-3 is required for GO-induced apoptotic signalling in AML. In this paper we showed that caspase-2 inhibition by the caspase-2 inhibitor z-VDVAD-fmk blocked both GO- and calicheamicin-induced caspase-3 activation in HL60 cells. Thus, we demonstrated that caspase-2 plays a role in GO-induced apoptotic signalling and at least in part acts upstream of caspase-3. Since daunorubicin represents one of the standard chemotherapy drugs in AML treatment, we wanted to know whether it affects the same apoptotic signalling as was shown for GO. In HL60 cells we observed that proliferation was blocked, induction of apoptosis was induced and activation of caspase-3 was increased after 48h daunorubicin treatment (100 and 500nM). Unlike in the experiments above, HL60 cells subjected to daunorubicin in

combination with the caspase-2 inhibitor z-VDVAD-fmk, did not show a statistically significant reduction of caspase-3 activity, only a slightly reduction. This suggests that daunorubicin-induced apoptosis does not include activation of caspase-2 but involves caspase-3 activation through an alternative path.

As been shown earlier, the Bcl-2-family members Bak and Bax are important for mediating apoptosis through mitochondrial pathway [114] and we found in *paper I* that GO-treatment causes increased Bak and Bax activation in GO-sensitive cells but not in resistant AML cells. We therefore examine if caspase-2 is essential for Bak/Bax activation (Figure 1).

Importantly, we found that inhibition of caspase-2 activity with z-VDVAD-fmk did not affect activation of Bak and/or Bax after GO treatment and appeared, accordingly, not to induce mitochondrial depolarization. The same finding was observed when GO was replaced by calicheamicin, etoposide or daunorubicin. Thus, at least in these AML cells caspase-2 signalling in response to these drugs is via a non-Bak/Bax mediated route to caspase-3. Another Bcl-2-family member, Bid, is a BH3-only protein which is exclusively cytosolic in living cells and is cleaved during apoptosis into a truncated form, tBid, which subsequently translocates to the mitochondria [128] were it regulates Bax relocalization to the mitochondria leading to cytochrome-c release [129] [130]. By studying GO-induced Bid activation and the possible involvement of caspase-2, we found a dose-dependent decrease of full-length Bid after treatment with GO. Importantly, blocking caspase-2 only caused a slight reduction in Bid cleavage, hence we cannot see any clear role of this caspase in Bid processing after GO treatment. Thus, our data indicate that there are at least two pathways that are activated in AML cells in response to DNA-damaging agents, one involving Bak/Bax activation and another involving caspase-2 both which are of importance for implementing of apoptosis via caspase-3 in response to these agents (Figure 1).

We next analysed signalling events upstream of Bak/Bax and caspase-2 activation. For that purpose pulsed field gel electrophoresis (PFGE) was used to analyze DNA double strand breaks (dsbs), which are thought to be critical for the cytotoxic effect of GO. Calicheamicin has been reported to cause DNA dsbs [122] yet the involvement of DNA dsb in response to GO at clinically relevant doses remained elusive. In line with previous results we observed that free calicheamicin, already after 4h showed a 10-fold increase in formation of DNA dsbs, which remained also after 24h. After GO treatment we observed a 2-fold higher level of DNA dsbs at 24h but with no major increase at 1h or at 4h. This suggests that GO at clinically relevant doses does indeed induce DNA dsb, an event that coincides with caspase-2 activation.

In addition, to cause DNA dsbs, GO responsiveness has also been linked to induction of G_2 arrest in AML cells [113]. In line with previous reports we found that GO treatment during 24h resulted in an accumulation of 14% of the HL60 cells in G_2/M-phase (compared to 5% of the untreated cells). Importantly, analysing the role of caspase-2 in this context showed that inhibition of caspase-2 activity with z-VDVAD-fmk did not alter the cell cycle distribution. Therefore, our results suggest that to affect the cell cycle arrest due to DNA damage induced by GO, the catalytic activity of caspase-2 does not seem to be required. Thus, caspase-2 seems to be required only for apoptotic signalling.

To reach and maintain complete remission (CR) are important features for the clinical outcome in AML. In an attempt to place these cell line-derived results in a clinical context, we also analysed the expression of caspase-2 and caspase-3 in blast cell populations isolated from 22 AML patients, from the above-mentioned patient cohort, at the time of diagnosis, prior to therapy. Full-length caspase-2 (n=17) and caspase-3 (n=19) were examined in mononuclear cells and the patients were divided into two groups according to CR duration (short CR <6 month and long CR >6 month). Caspase-2 or caspase-3 protein expression varied among patients, with a greater spread in the group with long CR duration. Caspase-3 did not show any differences in expression while for caspase-2 we observed a higher amount in patient with long CR duration. However, an analysis of a larger patient cohort including full length and cleaved caspase-2 could likely better clarify if caspase-2 expression is divergent already at diagnosis. Such analyses are on the way.

In conclusion, we demonstrated that GO caused a cleavage of caspase-2 in GO-responsive AML cells but not in GO-resistant AML cells. Thus, caspase-2 has a critical role in the intracellular cell death signalling after GO treatment in AML cells. Our results suggest that caspase-2 probably works in a pathway separate from the intrinsic pathway, leading to activation of caspase-3, as blocking of caspase-2 did not alter the pro-apoptotic events linked to Bak, Bax or Bid.

4.3 PAPER III

Gene expression analyses at time of diagnosis indicate biomarkers predictive of therapeutic response in acute myeloid leukemia

In *paper III* we aimed to identify biomarkers predictive of clinical response in AML. For that purpose we isolated mRNA from diagnostic samples from 42 AML patients ("training cohort") which were subjected to Affymetrix® gene expression analysis (Figure 2). All patients entered CR after high-dose induction chemotherapy, reaching a median CR duration of 161 (range 12-3701) days. Samples from patients with "short CR duration" (<6 months, n=24) and "long CR duration" (>6 months n=18), respectively, were pooled together and a comparison of gene expression in these cohorts was examined.

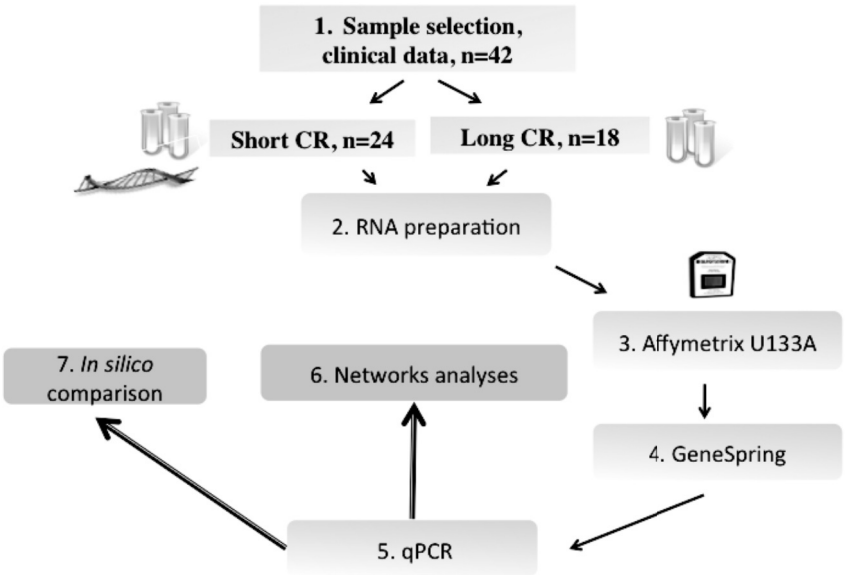

Figure 2: Workflow of gene expression analysis in AML patients (n=42)

We found a clear difference in gene expression at diagnosis in AML patients, when comparing the two clinically different groups and analyses revealed 383 genes to be up-regulated and 610 genes down-regulated more than two fold in samples from patients with short, as compared to those with long CR duration. Out of these genes the most prominently regulated was runt-related transcription factor 1; translocated to 1 (cyclin D-related) (RUNX1T1), which was up-regulated 116 fold in patients with short CR compared to those with long CR. The Ca^{2+}-dependent phospholipid binding protein Annexin 1 (ANXA 1) was down-regulated 58 fold in the same comparison.

We next validated the observed gene expression alterations with RT-qPCR and

could show that RUNX1T1, TKTL1, U2AF1 and NUDT4 all had a higher expression in patients with short CR duration, compared to those with long CR duration, whereas ANXA 1, FLRT3 and TLR8 were expressed at lower levels. Next we wanted to ensure that no single patient was behind the observed large up-regulation of RUNX1T1 in the group with poor responders and therefore we examined RUNX1T1 mRNA expression in individual AML patient samples in each group (Figure 3). Albeit RUNX1T1 mRNA expression varied among the individual patients a significantly higher transcript levels of RUNX1T1 were confirmed in the poor outcome group when performing RT-qPCR on individual samples (n=20).

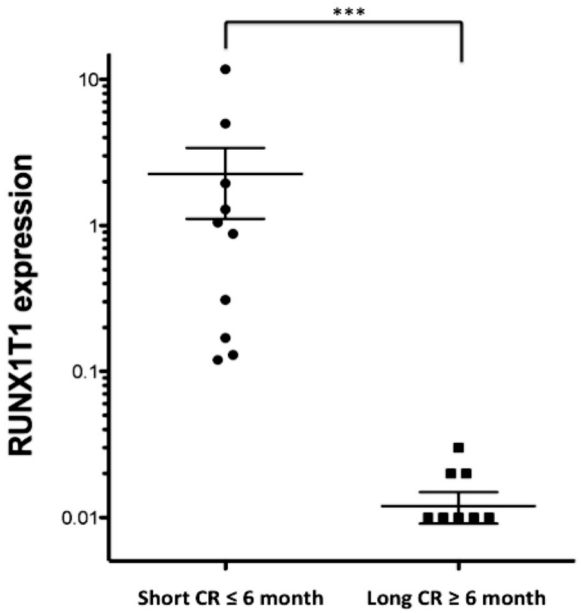

*Figure 3: RT-qPCR analysis of RUNX1T1 gene expression in individual patient samples (n=20). Loading control: GAPDH. Statistical analyses: Wilcoxon rank-sum test. ***p=0.0002.*

To identify potential signalling networks and their respective functions associated with these top up- or down-regulated genes in the short CR duration group of AML patients and their respective functions, IPA network analysis was carried out. The top functions revealed were cell death, developmental disorders, genetic disorders, metabolic disorders, cancer and haematological disease. We next focused on RUNX1T1 and applied IPA to reveal putative interaction partners. Interestingly, we found that most of its interacting partners were transcription factors and one of these was transcription factor 3 (TCF3), which indeed was found to be up-regulated in patients with short CR duration (5 fold). Another partner of RUNX1T1 with a higher expression in poor responders was the cluster of differentiation molecule 34 (CD34), which showed a 3 fold up-regulation. CD34, a cell surface antigen, is reported to be selectively expressed on both normal and leukemic human hematopoietic progenitor and stem cells [131] [132] and is also been demonstrated to be a predictor of poor

prognosis in NPM1-positive AML patients [133] [134]. Moreover, an association between CD34 expression and the expression of multidrug resistance gene (MDR1) has been revealed [135], and is suggested to be a contributing factor to the reason for the adverse prognosis in CD34-positive AML patients [136]. Thus, with network analyses, we found that RUNX1T1 indeed was associated with a number of transcriptional regulators and of these TCF3, also was up-regulated in AML patients with short CR duration.

To validate our gene expression results in independent patient cohorts we made an *in silico* comparison of two larger AML patient cohorts. For the *in silico* validation, training and validation cohorts were analysed using GeneSpring GX10 software comparing patients with poorer to those with better outcome. Comparative analysis was performed in each group (i.e. patients with short CR duration/OS vs. long CR duration/OS) and the results from each group were then compared with the results from the other groups in terms of up- or down-regulated genes. To gain a suitable amount of regulated genes we chose to set the cut off to 1.3 fold. These analyses resulted in 52 genes, which were found to be regulated in all three cohorts (Figure 4).

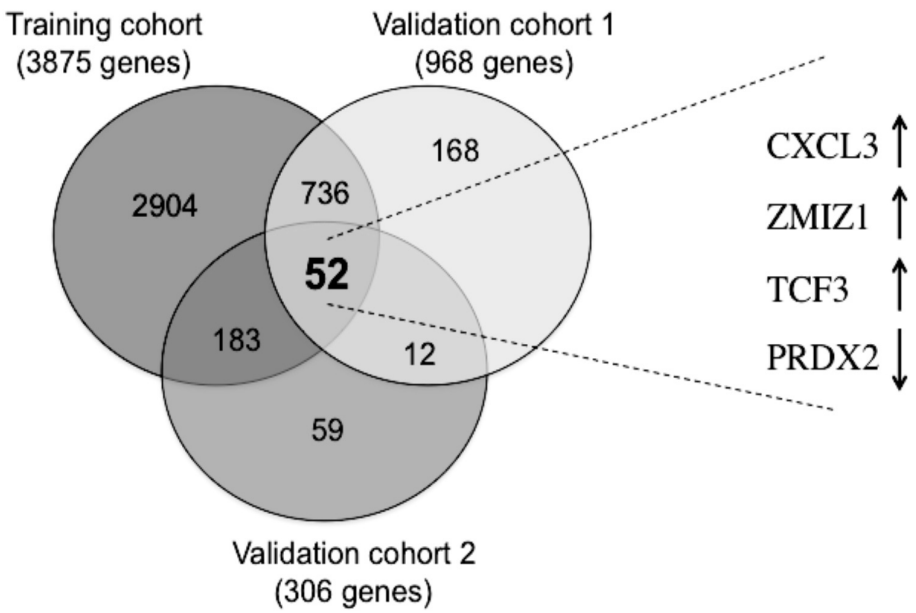

Figure 4: Venndiagram showing regulated genes in each cohort, respectively. The training cohort gene array results from pooled patient samples (n=42), validation cohort 1 gene expression results from Metzeler et al [117] (n=163) and validation 2 gene expression results from Raponi et al [118] (n=34). Cut off set to 1.3 fold. From the similarly regulated genes from all three cohorts further stratification were done according to cancer, leukemia, apoptosis and proliferation and revealed four genes (to the right).

Reducing the analysis to genes previously reported to be involved in cancer, leukemia, apoptosis or proliferation made further limitations of the genes. With this approach we ended up with three genes, Chemokine (C-X-C motif) ligand 3 (CXCL3), Zinc finger, MIZ-type containing 1 (ZMIZ1) and TCF3 up-regulated and Peroxiredoxin 2 (PRDX2) down-regulated in the group with poorer prognosis. CXCL3 expression has previously been shown to be increased in colon cancer, as compared to normal tissue and were demonstrated to be higher in patients with local versus systemic disease [137]. Given that we see a higher expression in patients with short CR/OS, we hypothesize that high expression of CXCL3 in leukemic blast cells may improve development and maintenance of leukemia. ZMIZ1 is a transcriptional coactivator of the protein inhibitor of activated STAT (PIAS) family of proteins [138]. Its role in oncogenesis is unknown. Rakowski and colleges, however, have shown that ZMIZ1 functionally interacts with NOTCH1 to promote transcription and activity of c-MYC and that silencing of ZMIZ1 delayed tumor growth and increased apoptosis in a T-ALL cell line [139]. The function of ZMIZ1 in AML is unclear, but our results are in line with the findings of Rakowski et al, since ZMIZ1 was up-regulated in all three cohorts. TCF3, one of the transcription factors that interact with RUNX1T1, was found to have a slightly increased expression in patients with poorer prognosis also in validation analysis. In colorectal cancer cells overexpression of TCF3 has been shown to repress induction of Wnt signalling which subsequently leads to increased proliferation [140]. This is in line with our observation demonstrating a higher expression of TCF3 in AML patients with poor prognosis, which would possibly lead to an increased proliferation of leukemic blast cells. PRDX2 is a gene with an antioxidant function in cells [141] and has interestingly been identified as a tumour suppressor gene with reduced expression in AML [81]. In validation of our results we found PRDX2 to be down-regulated in all three cohorts albeit to different degree.

Analyses of overall survival in relation to mRNA expression of selected genes (CXCL3, ZMIZ1, TCF3 and PRDX2) were conducted (Validation cohort 1) by dividing the patients according to high or low gene expression. Cut off were set to the median value of gene expression and survival data added to Kaplan-Meier plot. Regarding ZMIZ1, we found a significantly shorter survival in patients with higher ZMIZ1 expression ($p=0.03$), which could not be shown in relation to CXCL3. In contrast, TCF3 showed a lower expression in patients with poorer prognosis. There was no difference between low or high expression of PRDX2 regarding survival.

All in all, our results show that by analysing gene expression in AML patient samples according to clinical outcome, one may indeed identify gene expression alterations which could hold biomarker potential of CR duration in this tumour malignancy. Thus, we showed that three genes, CXCL3, ZMIZ1, TCF3, involved in tumour genesis were up-regulated in patients with poor prognosis and that one gene, PRDX2, associated with tumour suppression was down-regulated in the same group of indicating that these or their associated signalling networks may be of biomarker value.

4.4 PAPER IV

Multidrug Resistance in Relapsed Acute Myeloid Leukemia: Evidence of Biological Heterogeneity

A major challenge in treating AML is resistance to chemotherapy. Multidrug resistance (MDR) is achieved when a single drug treatment causes the development of resistance to other unrelated drugs [73] and MDR is thought to be one mechanism behind drug resistance of AML [74] [142] [143]. In this study we wanted to assess 380 genes, chosen based on their potential role in MDR, in paired samples (at diagnosis and after relapse) from AML patients. The methods used is shown in Figure 5.

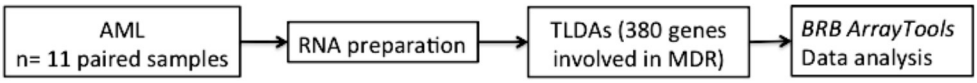

Figure 5: Methods used in paper IV. Paired samples from 11 patients (at diagnosis and at time of relapse). TLDAs: TaqMan Low Density Arrays, MDR: multidrug resistance, BRB ArrayTools: a microarray-data statistical analysis tool.

Taken into account that AML is a heterogeneous disease the interpatient variation was reduced by making a patient-to-patient analyses of diagnosis and relapse sample. By this approach the risk of false hits, which is related to AML heterogeneity, was decreased. "Unsupervised hierarchical clustering" showed that 6 out of 11 paired samples were grouped together suggesting that the resistance development was not likely caused by a major genetic change between the different time points. In contrast, the other five patients had a large heterogeneity in their gene expression observed in paired samples suggesting that the leukemic blasts at relapse had a different origin compared to at diagnosis. Moreover, in samples that clustered apart there was not any trend when correlated to FAB-classification, treatment, CR duration or OS, which may be due to the heterogeneity of the disease or to a small number of patients analysed.

To find out whether the MDR genes are reflecting the maturation stage of the AML cells, we correlated diagnostic samples with FAB class (M0-M5), which reflects the degree of maturation of the leukemia cells. Interestingly, this revealed that 52 out of 331 genes were significantly associated to FAB. Bcl2-related protein A1 (BCL2A1) and glutathione reductase (GSR) were two genes ($p<0.05$ and FDR <15%) in which gene expression was increasing with the maturation of the cells, i.e. correlated to FAB class M0 to M5. Moreover, four genes showed a negative correlation with high expression in immature cells compared to more differentiated cells i.e. DNA polymerase eta (POLH), H/ACA snoRNPs (small nucleolar ribonucleoproteins) gene family (NOLA2), ATP-binding cassette sub-family D member 4 (ABCD4) and CDK-activating kinase assembly factor MAT1 (MNAT1). As CR duration is of importance for prognosis and survival in AML, we also correlated diagnostic samples to CR duration. Out of the 331 genes examined 38 genes were significantly correlated with

CR duration but none of them reached FDR <15% possible due to a low number of patients.

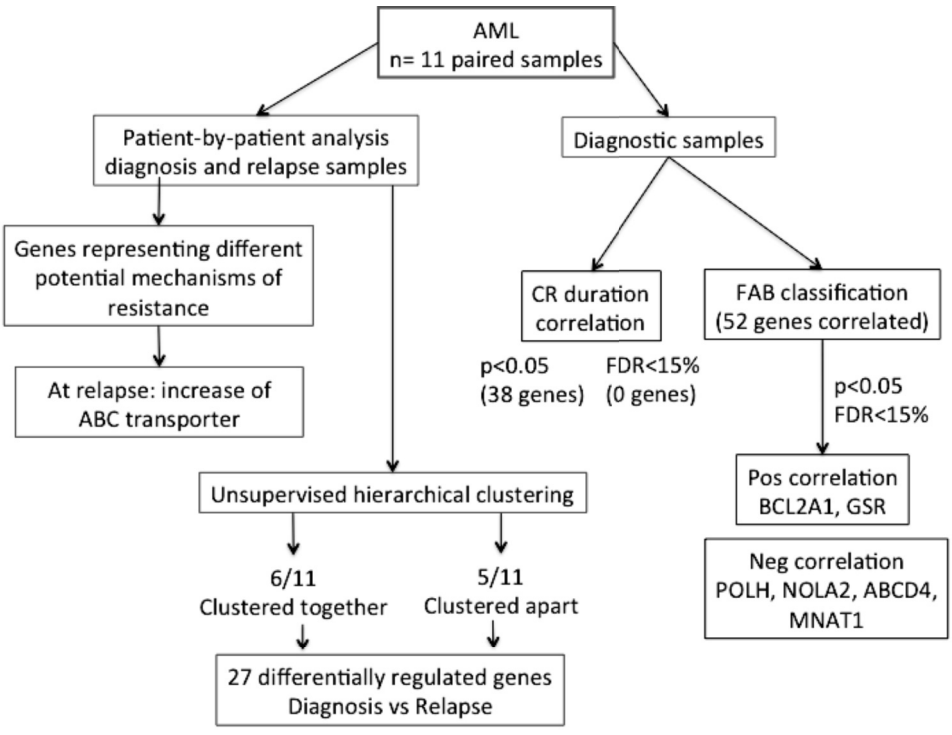

Figure 6: Overview of analysis in paper IV, according to analysis made from diagnostic samples or from paired samples (i.e. diagnostic and relapse sample).

Interestingly, analysis of paired (patient-by-patient) samples revealed that each patient had a unique gene signature likely reflecting that diverse resistance mechanisms are potentially operative in different AML patients. Focusing on nine ABC transporters which previously has been shown to mediate a MDR phenotype in other tumour types [73] [144] [145], we found that all patients except one, expressed an increase of at least one ABC transporter, at time of relapse. Importantly, these transporters were capable of transporting anthracyclines, vinca-alkaloids or both [146] [77]. Furthermore, one patient was found to have an overexpression of four ABC transporters i.e. ABCB1, ABCC1, ABCC5 and ABCG2 indicating that chemotherapy including anthracyclines or vinca-alkaloids will probably not benefit this patient [73]. In conclusion, we found that six patients out of 11 showed a similar MDR associated gene expression pattern at diagnosis and relapse suggesting that the same leukemic clone may be responsible for relapse. In contrast, in the other 5 patients analysed, the MDR-linked genes showed great difference at diagnosis and relapse indicating that in this subset of AML patients, a MDR phenotype may be driven by altered expression of one or several different ABC transporters. Our results indicates the importance of making repeated analyses of gene expression in order to consider further treatment for each individual patient.

5. DISCUSSION AND FUTURE PERSPECTIVES

5.1 PAPER I-II

Apoptotic signalling include intrinsic and extrinsic pathways as a response to GO treatment in AML cells

GO has recently attracted renewed interest as a targeted therapy for AML. The specific cellular and molecular mechanisms induced by GO treatment in AML are not fully understood and further analysis may help us to understand. Therefore we analysed response to GO-induced apoptotic signalling in AML cell lines and patient-derived AML cells treated *in vitro*. Primary AML cells were analysed to verify that the results from cell line analyses were also relevant in patient cells and for which AML patient it may be of value, thereby making translation of our *in vitro* findings into clinic. In conclusion we demonstrate that GO induce activation of caspase-3, Bak/Bax and p38 MAPK in GO-sensitive cells but not in GO-resistant cells (*paper I*). Focusing on the role of caspase-2 in GO or daunorubicin induced apoptotic signalling we show that GO cause caspase-2 cleavage into active fragment (*paper II*). When blocking caspase-2 we demonstrated a decrease of caspase-3 activation in GO-sensitive cells (*paper II*). Thus, our results implicate that CD33 expression, DNA damage signalling and repair, pro-apoptotic pathways as well as p38 MAPKs are involved in the sensitivity and resistance to GO and other chemotherapies of AML. Below are some reflections of these findings and future directions on how our findings may be of value for novel therapeutic strategies for AML.

In *paper I*, we demonstrate that HL60 cells and CD33-positive primary cells isolated from AML patients at diagnosis, showed a clear dose- and time-dependent effect of both GO and calicheamicin alone at clinically relevant doses (10 to 1000 and 0.3 to 30 ng/ml, respectively). The necessity of AML blast cells to express CD33 in order to respond to GO treatment has been discussed before. Although it would be reasonable to believe that the CD33-positive cells would be more sensitive to GO in contrast to CD33-negative cells, Boghaert and colleagues showed that an accumulation of a conjugate of anti-CD33 and calicheamicin in human tumour xenograft in nude mice, in the absence of detectable amounts of targeting antigen, led to sufficient accumulation of the drug to inhibit tumour growth of 10 different CD33-negative xenograft models [147]. Other *in vitro* studies have also revealed a direct relationship between CD33 expression and GO-induced cytotoxicity [148]. Data obtained from correlative studies conducted within the context of GO clinical trials for adult relapsed AML, are suggesting that CD33 expression may be associated with other AML prognostic factors [89] [149]. All in all our results and others correspond with the notion that the clinical effect of GO treatment for AML patients, in sufficiently high doses, cannot fully be linked to the degree of CD33 positivity in individual leukemic cell populations [89] [90] [150].

The results on GO presented in this thesis, as well as with daunorubicin illustrates that failure to activate pro-apoptotic signalling at mitochondria could impart chemotherapy response of AML. When the pro-apoptotic effector proteins Bak and Bax are activated they form pores in the outer mitochondrial membrane leading to mitochondrial outer membrane permeabilization and release of cytochrome C that

promotes caspase activation [114] [151]. In *paper I* we showed that a functional GO-induced apoptotic response involved proper activation of Bak and Bax in GO-sensitive, but not in GO-resistant KG1a cells. Thus in *paper I,* we speculate that one resistance mechanism of GO may be located up-stream of Bak/Bax. In order to find a protein responsible, or in part responsible for this activation, one way is to further analyse the BH3-only protein Bid and its truncated form (tBid). BH3-only proteins are known to be part of the activation of Bax and communicating in both the extrinsic and intrinsic pathway. To perform pro-apoptotic function, BH3-only protein requires the presence of Bak and Bax proteins [99]. In *paper II* we demonstrated a decrease of full-length Bid in response to GO treatment, but further analyses are needed including simultaneously assessment of the tBid increase and Bax activation to reveal if tBid is instrumental in causing GO-induced Bax or Bak activation. The Bcl-XL prevents apoptosis in hematopoietic cells [152] and is reported to be abundantly expressed in both megakaryocytes and erythrocytes [153]. Moreover, one may speculate that Bcl-XL could function by binding to activated Bak and Bax so that they can no longer oligomerize [99] [154].

Moreover, it would therefore be interesting to further analyse Bcl-XL in AML cells lacking Bid cleavage and activation of Bak/Bax, to reveal if Bcl-XL could imparting on these events. A high expression of Bcl-XL may also include a simultaneous block of caspase-2 induced apoptosis as previously been reported [155]. In the context of our findings on GO-induced caspase-2 cleavage it would therefore also be interesting to analyse if Bcl-XL in this aspect, also in our AML patient material, may be linked to caspase-2 expression and CR duration in the clinical setting.

Gemtuzumab ozogamicin (GO) is a remarkable therapeutic agent that is not yet easily available on the market. Although a multitude of clinical and molecular studies have been performed, there is still no unequivocal evidence regarding the benefit-risk ratio of this drug. Our results on GO-induced signalling *in vitro* support that there are patients clearly sensitive to GO and who probably would benefit from this treatment. Our apoptosis signalling data may help to further select these patients.

5.2 PAPER III-IV

Gene identification of CR duration biomarkers and multidrug resistance mechanisms in AML patients based on gene expression analyses

AML is a highly heterogeneous disease and the effectiveness of chemotherapy in AML varies among individual patients. In the last decades rapid development in omic technologies i.e. gene expression, proteomic profiling and DNA and/or RNA sequencing development as well as in computerized bioinformatics tools enable a global analysis of molecules aberrations. Thus, it has become possible to link certain molecular feature of an individual patient tumour to treatment response and in this way allow a more tailored treatment approach as exemplified by the presence of t(15;17)(q22;q12) in APL, where the patients are treated according to specific guidelines due to a superior treatment response. Unfortunately, AML biomarkers that could guide treatment remains to be elucidated.

In an attempt to identify such biomarkers that are significantly associated with remission duration in AML, we analysed gene expression followed by pathway analysis (*paper III*). We found that gene expression varies widely between the AML patients with short and long CR duration. In pooled cell samples from patients with short CR duration, as compared to those with long, gene array analysis revealed 10 genes to be up-regulated and 5 genes down-regulated >30 times. The transcription factor gene RUNX1T1, located on chromosome 8q22, was the most prominently up-regulated (x116) in the group with the short CR duration as compared with long CR duration. RUNX1T1 is known to be activated in AML including the M2 subtype and chromosomal translocations involving this gene are well documented in AML [3]. Thus one of the most frequently observed cytogenetic aberrations in AML is t(8;21)(q22;q22), which involves a fusion between RUNX1T1 and RUNX1 which give rise to a fusion protein [156]. Patients with this specific translocation have been considered to have low risk leukemia, according to cytogenetic aberration [22]. In our study we found only three patients expressing this translocation. Still, RUNX1T1 was markedly, highly up-regulated in patients with poorer prognosis. It is tempting to think that a high expression of RUNX1T1, without this specific translocation (i.e. t(8;21)), may have other effects on cell signalling or development and maintenance in AML. Thus, we know that RUNX1T1 interact with DNA-bound transcription factors and recruit co-repressors, such as mSin3, N-cor, HDAC1, to facilitate transcriptional repression [157]. With this knowledge, one can speculate that there may be other interactors, such as tumour suppressor genes, where RUNX1T1 can contribute to increase the transcriptional repression and thereby accelerate tumour growth or proliferation.

In IPA we analysed networks focusing on RUNX1T1. Interestingly, among the interacting partners of RUNX1T1, we found that most of them were transcription regulators. As a controller of the flow of genetic information from DNA to mRNA, transcription factors may be of importance in the gene expression of leukemic cells. In our study IPA revealed TCF3 as one of the interacting partner of RUNX1T1 and was also slightly up-regulated in training and validation cohorts. In breast cancer, Slyper et al, observed that expression of TCF3 was linked to poor tumour differentiation in basal-like subtype, and that high TCF3 levels were significantly associated with poor survival [158]. Moreover, as a repressor of Wnt signalling target genes, TCF3 is suggested to block the ability of butyrate to enhance Wnt activity and therefore contribute to colorectal cancer cell tumour genesis [140]. Further analysis of TCF3 is therefore warranted.

Mutation analyses have an enormous dynamic evolution when it comes to analyses of AML relapse [159]. Walter and colleagues discusses that myelodysplastic syndrom evolving into AML are driven not only by recurrent mutations from founding clones but also from the daughter subclones [159]. In RUNX1 (i.e. a translocation partner of RUNX1T1), seven acquiring novel mutations has been demonstrated in *de novo* AML with non-complex karyotype, associated with an unfavourable prognosis [160] and linked to chemo therapy resistance [161]. When studying the benefit and clinical relevance of RUNX1 as a marker for residual disease, Kohlmann and colleagues described a smaller subset of patients in relapse were the initial mutation was not detected but in which the occurrence of a novel mutation of RUNX1 was evident [162]. This may be due to an evolution of a novel resistant clone. Albeit mutations in

RUNX1T1 is not described in AML, there a few reports of somatic mutations in other cancers, such as breast-, lung- and colon cancer, suggesting these mutations to have functional effects and may function as oncogenes [163] [164]. Hence further studies in RUNX1T1 in AML are encouraged.

In validation of the gene expression from the training cohort, we could not verify the high expression of RUNX1T1 in patients with poor prognosis. This may be due to a small number of patients or possible the lack of subgroup analysis. Interestingly, we found 52 genes regulated in all three cohorts and of those four genes aroused a special interest according to their reported involvement in cancer, leukemia, apoptosis or proliferation. One of the identified genes was CXCL3. CXCL3 is a member of the growth-related oncogenes (GRO) and binds to a receptor, CXCR2 [165]. CXCL3 has previously been described in whole blood from healthy donors [166] and the blood cells were also the main synthesis sites of its receptor CXCR2 [166]. However, CXCL3 has previously only been connected to solid tumours. Thus in patients with colon cancer, an increased expression of CXCL3 as compared to normal colon tissue was described, and it was shown that the CXCL3 expression level was significantly higher in patients with local versus systemic disease [137]. Doll and colleagues also suggested that CXCL3 together with other chemokines (e.g. IL-8) accompanies the induction of metastasis in colorectal cancer, but might not be necessary for maintenance of the metastatic disease [137]. Moreover, in estrogen receptor-α positive breast cancer, high expression of CXCL3 was correlated to significantly shorter relapse-free survival [166]. As in the former situation, we observed an up-regulation of CXCL3 in AML patients with poorer prognosis, but the effect of CXCL3 in AML still needs to be elucidated.

Another gene we found to be up regulated was ZMIZ1. ZMIZ1 is reported to contribute to c-MYC activation and oncogenesis by collaboration via activated NOTCH [139]. c-MYC function is essential for proper haematopoiesis as it regulate the balance between self-renewal, differentiation and proliferation that is required for blood formation. Interestingly, c-MYC is reported to have an increased expression in certain cases of AML where it is thought to interrupt the balance in haematopoiesis leading to increased proliferation and simultaneous blocking terminal differentiation [167]. In our study we observed higher expression of ZMIZ1 in patients with poorer prognosis. Due to earlier described activation of c-MYC and collaboration ZMIZ1-NOTCH, one can imagine that silencing of ZMIZ1 could have an impact even in AML blast cells slowing tumour growth and increasing apoptosis as described in T-ALL cells [139]. Analyses on AML cells still need to be performed, but one can think that, if this is true for AML cells, there is a possibility to use ZMIZ1 inhibitors as a part in the treatment of AML.

The only gene that we found in *paper III* to have a decreased expression in AML patients with short CR/OS was PRDX2, an antioxidant that acts as an inhibitor of myeloid growth [168] [81]. Agrawal-Singh et el, has described PRDX2 as a tumour suppressor gene, as a low expression level was clinically associated with poor prognosis in AML [81]. Histone acetylation and DNA methylation patterns are marks that ensure accurate transmission of chromatin states and gene expression patterns and their interplay may involve gene silencing in tumours [82]. Arawal-Singh et al, have investigated the histone H3 acetylation in several loci in the AML epigenome and found that PRDX2 was silenced by epigenetic mechanisms as a consequence of DNA

hypermethylation and loss of H3 acetylation at the promoter region [81]. A role for PRDX2 as a tumour suppressor gene is in line with our findings where significantly lower expression of PRDX2 was found in patients with poor prognosis. Interestingly, it is suggested that PRDX2 act as a growth suppressor in leukemia induction, which is caused by the c-MYC oncogene [81], a mechanism previously shown in T-ALL cells upon ZMIZ1.

In conclusion, we found that the ZMIZ1 gene was up regulated in AML patients with poor prognosis, which might be due to its connection to c-MYC. Ideally an inhibitor of ZMIZ1 would be interesting to examine for its capacity to decrease proliferation of leukemic blast cells but as such not is available and alternative approach would be to use inhibitors towards the transcription factor c-MYC which recently has been described [169]. The decreased expression of the tumour suppressor gene PRDX2 linked to poor prognosis in AML might be used as a predictor of clinical outcome in AML.

Dealing with chemotherapy resistance in AML patients is a big challenge. A majority of the AML patients will have a recurrent disease within the first two years after diagnosis and the treatment response after relapse is poor. How to circumvent resistance to therapy is clinically relevant and has attracted a lot of interest. Despite that, there is still a great need of knowledge. For chemotherapy resistance in AML as well as in other tumour types a number of signalling aberrations are evident such as decreased drug-uptake, activation of DNA-repair mechanisms and evading drug-induced cell death e.g. apoptosis [73] [170] [74].

In *paper IV* we took a global approach and assessed if 380 previously MDR-related genes [120] had a different expression in AML samples from the time of diagnosis and at relapse. Performing unsupervised hierarchical clustering on paired samples from 11 individual AML patients, we found that five pairs were clustering apart and thus demonstrated a change in gene expression at time of relapse as compared to diagnosis. The genes that differ in expression may provide insight into what contributes to the development of chemotherapy resistance in AML and why AML at relapse are more resistant to treatment. It has earlier been shown that MDR1 (i.e. ABCB1) usually are present in a low level in AML at time of diagnosis [74], which corresponds well with a good response of induction treatment. Moreover, MDR1 is also demonstrated to have an increased level at relapse [142], supporting a poorer response to therapy at relapse. The expression level of MDR1 seems to increase with age [42] and in some studies it has been found to be predictive for poor outcome [171] [172] [74]. MDR1 is also reported to have an increased level in secondary leukemias [142]. One can think that genes contributing to relapse may change their mutation status thereby altering their function and in this contribute to a chemotherapy refractory phenotype.

There was an enormous heterogeneity in gene expression in accordance to FAB classification, but for samples taken at diagnosis, two genes showed a positive correlation and four genes a negative correlation to maturation status in AML cells. Genes expressed in diagnostic samples and which showed a correlation to CR duration would be of interest since those genes may be one of the reasons for relapse or a predictor of recurrent disease. Our study did not reveal any significant relations that fulfilled FDR <15%. This may be due to a small amount of samples. It is also possible

that there are genetic aberrations that in fact is appearing after induction treatment and leading to a new cytogenetic signature compared to the one found at diagnosis.

In support of the above, is the study by Walter et al, who reported that the development of secondary leukemia is dynamic and processed by multiple cycles of mutations and clonal selection [159]. They suggest that not only the presence of recurrent mutation, but also by the clone from which they arise may contribute to progression [159].

To study differences in disease progression at diagnosis and in relapse we made a patient-by-patient analysis to determine whether the individual patients gene expression pattern differed between the two time points. Remarkable, in each patient samples at the time of relapse, there was an enormous heterogeneity, where the expressed genes likely represent different mechanisms of resistance, which all may contribute to the recurrent disease. Interestingly, in five of the patients (in patient-by-patient analysis) we found an increased expression of MDR1 at relapse, as described above this gene is well described to contribute to relapse in AML, but the diverse expression of MDR genes among the patients makes it likely that there are different genes contributing to relapse in different patients or different subgroups of disease. The diverse outcome in molecular analyses when comparing diagnostic and relapse samples suggests that systemic chemotherapies and/or leukemic blast cell selection have a considerable influence on the clonal evolution. This means that personalized therapy is an important way to go to achieve continued improvement in therapy response, but also tricky. With a large amount of genetic data in each individual patient suggesting a possible effect on resistance or residual disease, one problem is to know which target or targets, is the most clinically relevant. A lot of clinical challenges still remain unsolved.

In summary, comparing AML patient cohorts with short vs. long CR duration our data demonstrate major differences in the RNA-expression of genes known to be involved in important regulatory events in normal and leukemic haematopoiesis. The differences are detectable already at diagnosis and may therefore be valuable in predicting AML outcome and for identification of new therapeutic biomarkers (*paper III*). In training cohort we found RUNX1T1 to be markedly up-regulated in patients with short CR duration, indicating that RUNX1T1 may signal poor long-term prognosis. Additional data from an unrelated validation cohort highlighted further differential genes of possible clinical significance, TCF3, CXCL3 and ZMIZ1 which were found to be up-regulated in patients with poorer prognosis and PRDX2 which was down-regulated in the same group (*paper III*).

By analysing 380 genes associated with MDR and correlated these to 11 paired AML patient samples, we revealed that 6 out of 11 patients clustered together indicating that leukemic blast cells at relapse had the same origin as in the first sample (*paper IV*). The other 5 clustered apart, where the leukemic clone cannot be traced back to the same origin, but are perceived to be a newly developed clone. Patient-by-patient analysis showed that 10 of 11 patients at relapse expressed an increased amount of ABC transporters that have been shown to mediate multidrug resistance (*paper IV*). Those findings underline the importance of individual molecular diagnostics for specific individual treatment.

6. SUMMARY AND CONCLUSION

Paper 1

Our results from GO-treated AML cell lines and primary AML cells pinpoint the importance, not only of CD33-expression but also of p38 MAPK activation with subsequent initiation of mitochondrial depolarization and caspase-3 activation, as molecular determinants for clinical GO responsiveness in GO-sensitive cells. None of the above events occurred in GO-resistant AML cells upon GO-treatment. We could also, for the first time, show that activation of Bak/Bax appeared to be required for GO-induced apoptosis and that resistance to GO may be located upstream of Bak/Bax.

Paper II

We demonstrated that, caspase-2 was cleaved in GO-responsive AML cells but not in GO resistant cells after *in vitro* treatment. Thus caspase-2 likely has a critical role in the intracellular cell death signalling after GO treatment in AML cells. Caspase-2 probably acts in an alternative route to caspase-3 activation that acts in parallel to the intrinsic pathway. Blocking caspase-2 did not alter pro-apoptotic activation of Bak, Bax or cleavage of Bid. For molecular markers, such as caspase-2 and caspase-3, it is desirable with further analyses of both full length and activated proteins to correlate with response to GO treatment. This is to find out whether there is an increased cleavage in responders and a higher expression after GO treatment. Cell lines are suitable for initial studies, but to make it clear that the results are useful in clinical situation, analysis of primary patient cells are needed.

Paper III

Gene expression analyses are powerful in finding important prognostic and/or predictive markers in AML. We compared AML patients with short CR duration (i.e. poor prognosis) to those with a long CR duration (i.e. better prognosis) and found a large difference in gene expression in the two groups. Our most striking finding was a remarkable up-regulation of the RUNX1T1 gene in patients with poor outcome. Pathway analyses linked RUNX1T1 to TCF3, which is reported to have tumour-initiating capacity in solid tumours and are involved in cell signalling leading to increased proliferation. By silencing TCF3 we might be able to decrease proliferation in AML cells. *In silico* comparison revealed ZMIZ1 to be up-regulated in patients with poor clinical outcome. This is a gene involved in tumourgenesis, probably due to indirect activation of c-MYC and this makes ZMIZ1 a possible target for personalized therapy by using a ZMIZ1 inhibitor. PRDX2, a tumour suppressor gene, was found to be down-regulated in AML patients with poor prognosis, thus a potential predictor of clinical outcome in AML.

Paper IV

We investigated the clinical relevance of 380 genes, known to affect the response to chemotherapy, in 11 paired samples (i.e. from diagnosis and at relapse) from adult AML patients. Hierarchical clustering revealed six patients clustering together, suggesting recurrence from the same leukemic origin, while 5 paired samples clustered apart. In patient-by-patient analyses of paired samples, we observed that all patients had a unique gene signature representing different resistance mechanisms. Those results pinpoint the importance of genetic and molecular diagnostics for personalized therapy.

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