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Gene expression profiling based identifications of potential prognostic molecular markers in decitabine treated acute myeloid leukemia

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Abbreviation:

A - adenine
ABL1 - Abelson murine leukemia viral oncogene 1
Ac - acetyl
AGCC – Affymetrix GeneChip Command Console
ALE – anti leukemic effect
ALL - acute lymphatic leukemia
AML - acute myeloid leukemia
AMLSG - German and Austrian AML Study Group
APL – acute promyelocyte leukemia
ASXL1 - sex combs-like 1
ATO – arsenic trioxide
ATRA – all-trans-retinoic acid
AZA – azacytidine
BCR - brealpoint cluster region
BAALC - brain and acute leukemia cytoplasmic
BCOR - BCL -6 co-repressor
BMAL1 - brain and muscle ARNT-Like 1
BSC – best supportive care
C - cytosine
CALGB – cancer and leukemia group B
CBF – corebinding factor
CDH1 - cadherin 1
CEBPA - enhancer binding protein alpha
CGI – CpG Island
CLL – chronic lymphocytic leukemia
CML – chronic myeloid leukemia
CMML - chronic myelomonocytic leukemia
CN – cytogenetic normal
CNS – central nervous system
CR – complete remission
CT – cycle threshold
CTCL – cutaneous T-cell lymphoma
DAC – decitabine
DAVID – database for annotation visualization and integrated discovery
dCK - deoxycytidine kinase
DEPC - diethylpyrocarbonat
DFS – disease free survival
DLBCL – diffuse large B-cell lymphoma
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DNMT – DNA methyltranferase
ECOG – Eastern Cooperative Oncology Group Performance Status

ELN - European Leukemia Net
EMA – European Medicines Agency
ERG - ETS related gene
EVI1 - ecotropical viral integration site 1
EXT1 - exostosis type 1
EZH2 - enhancer of zeste homolog 2
FAB – French-American-British
FDA – U.S. Food and Drug Administration
FISH – fluorescent in situ hybridization
FLT-3 - FMS like tyrosine kinase 3
G - guanine
GATA - globin transcription factor
G-CSF – granulocyte-colony stimulating factor
GEP – gene expression profile
GO – gene ontology
GVL – graft versus leukemia
H3K4 - histone 3 lysine 4
HAT – histone acetyltransferase
HCTCI – hematopoietic cell transplant co-morbidity index
HDACI – histone acetylase inhibitors
hENT - human equilibrative nucleoside transporter
HIDAC – high dose cytarabine
HKG – house keeping gene
HOX - homeobox
ID4 - inhibitor of DNA binding 4
IDH - isocitrate dehydrogenase
IHC – immune-histochemistry
ITD – internal tandem duplication
kbp - kilobase pair
LDAC – low dose Ara-C
LDH – lactate dehydrogenase
LS/KS - least squares/Kolmogorov-Smirnov
LSD1 - lysergic acid diethylamide 1
LSD1 - lysergic acid diethylamide 1
MAPK - mitogen activated protein kinase
MDS – myelodysplastic syndrome
MLL - mixed lineage leukemia
MN1 - meningioma 1
MPS – myeloproliferative syndrome
MRC - United Kingdom Medical Research Council
MSI2 – musashi 2
MUM1 – multiple myeloma oncogene 1
NGS – next generation sequencing
NPM1 – nucleophosmin

NOS - not other specified
NSD1 - nuclear receptor-binding SET domain protein 1
NUP98 - nucleoporin 98
NYHA – New York Heart Association
OMF – osteomyelofibrosis
OS – overall survival
PBMCs - peripheral blood mononuclear cells
PBS – panobinostat
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PD – progressive disease
PHF6 - PHD finger protein 6
PLK1 – polo-like-kinase 1
PML – promyelocyte leukemia
PR- partial remission
qRT-PCR - quantitative real time PCR
Qty - raw quantity
RARA – retinoic acid receptor alpha
RDS – romidepsin
RIZ1 - retinoblastoma-interacting zinc-finger protein 1
RNA – riboxynucleic acid
RBPJ – recombination signal binding protein
RT-PCR - real-time polymerase chain reaction
RUNX1 - runt related transcription factor 1
s-AML – secondary AML
SAGE – serial analysis of gene expression
SCT – stem cell transplantation
SD – stable disease
SNP - single nucleotide polymorphism
SOP - standard operating procedures
T – thymine
TAA – tumor associated antigen
t-AML - therapy related AML
TET2 - ten eleven translocation 2
TKD – tyrosine kinase domain
TP53 - tumor protein 53
TRPS1 -trichorhinophalangeal syndrome I
TSG – tumor suppressor gene
VPA – valproic acid
VST – vorinostat
WBC – white cell blood count
WHO – world health organization
WT1 - wilms tumor 1

1 Introduction

1.1 Acute myeloid leukemia

More than 160 years ago Rudolf Virchow was one of the first physicians who described the picture of leukemia by observing a large cell number of white blood cells in a chronic myelocytic leukemia (CML) blood sample and who termed this disease leukemia. Since then a lot of progress has been made to understand and to diagnose leukemia more precisely. Nevertheless, especially acute leukemia is still one of the least understood types of cancers with a high mortality rate.

The type of acute leukemia is defined by the cell line of origin, lymphatic, myeloid or undifferentiated. Additionally, characteristics like maturation state, etiology and cell count have to be considered. Acute lymphatic leukemia (ALL), which occurs more often in young children, and acute myeloid leukemia (AML), which is more common in elderly patients, are the most common ones. AML is defined as an uncontrolled clonal proliferation by myeloid blasts originating out of myeloid stem or progenitor cells in the bone marrow, thereby eliminating the physiological hematopoiesis. The clonal expansion of the blasts can also occur in other body tissues then so called chloroma. Due to the lack of healthy hematopoiesis or infiltration of other organs clinical symptoms occur.

1.1.1 Epidemiology:

The incidence of AML is around 3.7 per 100,000 persons in the Western world. There may be two peaks in occurrence young children below age of four and later adulthood, however the median age is 65 years [54]. The risk of developing AML rises about 10-fold from 30 to 65 years [156]. The chance for men to contract an AML is 1.5-fold compared to women [123]. Although the incidence of AML is rare, just <3% of all cancers, compared to other malignancies like colon, breast or lung cancer, it provides the leading cause of cancer related death in persons <39 years [54].

1.1.2 Etiology and risk factors:

Several risk factors have been found which seem to be associated with developing AML, however, known carcinogens only integrate for a small number of cases. These risk factors include irradiation, previous chemotherapy especially alkylating agents and topoisomerase II inhibitors causing a so called therapy-related AML (t-AML). Patients coming down with an AML, who have been treated with alkylating agents have an average latency period of about 5-7 years. Their diseases are often characterized with a complex karyotype featuring aberrations in chromosome 5 and/or 7 as well as a high incidence of tumor protein 53 (*TP53*) mutation [211].

Patients being predisposed to topoisomerase II inhibitors and generating an AML show frequently balanced translocations especially involving mixed lineage leukemia *(MLL),* nucleoporin 98 *(NUP98),* runt related transcription factor 1 *(RUNX1)* and retinoic acid receptor alpha (*RARA)* with quite shorter latency period (1.5-3 years) comparing to alkylating agents exposed AMLs [211].

Furthermore, exposure to viruses and chemical substances, such as benzene, have shown to increase the risk of developing an AML. On the other hand hematologic diseases like myelodysplastic syndrome (MDS), osteomyelofibrosis (OMF) and genetic disorders (Down syndrome, Shwachman syndrome, Fanconi anemia, Li Fraumeni syndrome etc.) are high risk diseases for developing an AML, called secondary AML (s-AML), presenting cytogenetic changes associated with their primary disease (for classification details see below) [54, 156]. In addition a few gene mutations have been associated with familial leukemia, however so far only *RUNX1* and enhancer binding protein alpha (*CEBPA)* germ line mutations have been identified with pure familial leukemia [177].

1.1.3 Clinical presentation:

The AML presents clinically very heterogeneously and with unspecific symptoms. These symptoms can occur pretty acutely and develop within a month. The clinical signs are mainly caused by bone marrow or other organ infiltration of the myeloid blasts, leading to a lack of normal leukocytes, erythrocytes and thrombocytes, respectively. Due to anemia common symptoms are paleness, fatigue, dyspnea and tachycardia. Some patients present with

petechia caused by thrombocytopenia. Besides the thrombocytopenia the lack of coagulation factors due to increased fibrinolysis activity caused by cytokine storms can often lead to bleeding such as spontaneous epistaxis or gingival bleeding [156]. Persistent fever without any signs of infections may also be considered, but on the other hand patients with neutropenia are also highly susceptible for infection like pneumonia or soft tissue infection leading to abscesses. Additionally, hyper leukocytosis with white blood cell counts > 100 G/l leukocytes may lead to leukostasis symptoms like ocular, cerebrovascular dysfunction, pulmonary distress syndrome or priapism. In the bone marrow this mass of blasts can cause bone pain likely from the increased pressure by the expansion of blasts in the bone marrow space. Other organ infiltrations like skin, spleen, liver or lymph nodes are the most frequent sites of extra medullary involvement. Several risk factors have been concluded with an increased risk of extra medullary leukemia including core binding factor aberrations like t(8;21), inv(16), expression of specific cell surface markers (CD65, CD2, CD4, CD7), French American British (FAB) subtypes (M2,M4,M5), age, peripheral high white cell blood (WBC) count, blast differentiation and maturation, lack of blast Auer rods and nutritional status [32]. If the cerebral spinal fluid is involved, which is seen in less than 5% of cases, patients may present with unspecific neurological symptoms like persistent headaches, dizziness etc. but can also be asymptomatic [15].

1.1.4 Diagnostics and Classification:

Over the last two decades the diagnostic procedures to determine the diagnosis of AML and its specific subtypes have advanced and have also changed the World Health Organization (WHO) classification. However, the primary diagnosis of AML still relies on morphology, the identification of myeloid blasts in patients' peripheral blood or bone marrow samples stained with Wright-Giemsa (Figure1).

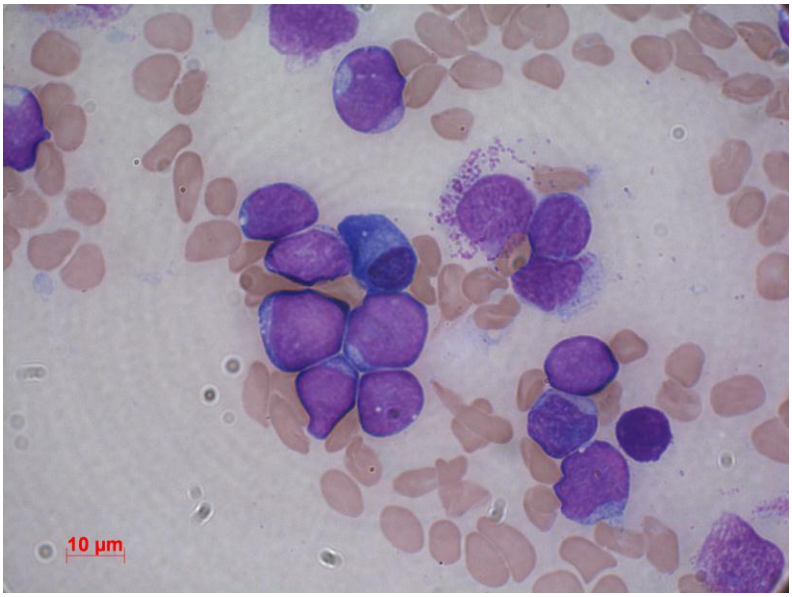

Fig.1: Infiltration of myeloid blasts in a bone marrow smear of a patient treated within this study

The presence of at least 20% leukemic blasts in the bone marrow aspirate is required with a few exceptions. The myeloid blasts must be distinguished from ALL, myelodysplastic syndrome (MDS) and AML evolving from a MDS. The distinction of AML to ALL and MDS rests for ALL on the different morphologic, immunohistochemical (IHC) and immunologic characterizations. However, for the MDS it requires additional clinical assessment and genetic analysis, especially in uncertain blasts count situations [155]. The morphology of the myeloid blasts within AML is very heterogeneous. Therefore, the variability has been used to determine particular morphologic subgroups. In addition, multiparameter flow cytometry is executed for the detection of immunophenotypic features that are crucial in approving the diagnosis and to specify the myeloid subtype (M0-M7). But there is no general agreement on the cutoff point for typical acute leukemia markers for being positive. For most of the markers expression on at least 20% of the cells is considered as being positive, but there are also selected markers with a suggested lower cut off point of only 10% [58]. Nevertheless, blast counts by flow cytometry are not supposed to replace morphologic blast counts. The French-American-British (FAB) classification divides the AML into eight major subgroups (M0-M7) relying on myeloid lineage involved and the degree of blast cell differentiation (Table1) [9].

Tab.1: The French-American-British (FAB) Classification [9].

FAB subtype	Description
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M4eos	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryocytic leukemia

Furthermore, over the last two decades the impact of cytogenetic and molecular genetic characteristics have been evolved and contribute essentially to the confirmation of the diagnosis of specific subtypes. These new accomplishments provide also insights into disease prognosis and therapy strategies. For an overview of chromosomal abnormalities chromosome banding analysis is the method of choice, whereas fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) use certain probes for submicroscopic mutation identification. How these more detailed diagnostic procedure affect therapeutic strategies can be nicely observed in a case of acute promyelocytic leukemia (APL). Defined by the genetic translocation t(15;17) leading to the rearrangement of the promyelocytic leukemia (*PML)* and *RARA* genes, and thereby causing an abnormal chimeric retinoic acid receptor transcription factor, the APL is now primarily treated with all-trans retinoic acid (ATRA), intravenous arsenic trioxide (ATO) plus anthracycline or cytarabine based chemotherapy [117].

All these different and essential analyses help to better determine the AML subtype. Therefore, all of them are accounted for in the World Health Organization (WHO) classification system for AML, which more heavily relies on the genetic changes underlying AML and puts more weight on them in the current version of 2008 (Table2) [224]. In addition to cytogenetics,

the growing importance of molecular genetics for the classification of leukemia is reflected in two new provisional entities defined by the presence of gene mutations in *nucleophosmin* (*NPM1*) and *CEBPA* [224].

Tab.2: WHO Classification 2008 of AML [224] (AML=acute myeloid leukemia;BCR-ABL1= brealpoint cluster region Abelson murine leukemia viral oncogene 1; CBFB= core binding factor beta CEBPA= enhancer binding protein alpha; EVI1= ecotropic viral integration site-1;MKL1=megakaryoblastic leukemia 1 MLL=mixed lineage leukemia, MYH11= myosin heavy polypeptide 11; NK= natural killer; NOS= not otherwise specified; NPM1= nucleophosmin, NUP214=nucleoporin 214 PML= promyelocyte leukemia ;RARA= retinoic acid receptor alpha; RBM15=RNA-binding motif 15; RPN1= ribophorin 1; RUNX1= runt related transcription factor; WHO= world health organization)

2008 WHO Classification of acute myeloid leukemia
Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
APL with t(15;17)(q22;q12); PML-RARA
AML with t(9;11)(p22;q23); MLLT3-MLL
AML with t(6;9)(p23;q34); DEK-NUP214
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
AML with mutated NPM1
AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified (NOS)
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blasticplasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); BCR-ABL1
Mixed phenotype acute leukemia with t(v;11q23); MLL rearranged
Mixed phenotype acute leukemia, B/myeloid, NOS
Mixed phenotype acute leukemia, T/myeloid, NOS
Provisional entity: Natural killer–cell lymphoblastic leukemia/lymphoma

1.1.5 Therapy:

So far, the therapeutic management of AML patients has been mainly based on the age and performance status of the patients due to potential high morbidity and mortality rate of about 10% for intensive induction therapy [134]. For patients younger than 60 years of age or patients with age adjusted good performance status of 2 or less are supposed to receive an intensive induction therapy. Recently, several study groups also started to treat patients based on a genotype-specific treatment approach. These new therapeutic strategies incorporate pharmacological inhibition of mutated or deregulated molecular targets and activated signaling pathways, as well as interaction with epigenetic regulation and myelosuppressive antibodies [204].

Even if the induction therapy has been relatively constant over the last twenty years, therapy within a clinical trial is recommended to guarantee the best evidence based medicine. The goal of the induction therapy is to achieve a complete remission (CR), which is defined by a bone marrow blasts count <5%, absence of blasts with Auer rods, absence of extramedullary disease, absolute neutrophil count > 1.0 G/l, platelet count >100 G/l and independence of red cell transfusions [58]. If complete remission is achieved, a postremission therapy, a consolidation therapy, is followed. Compared to induction therapy, for post-remission therapy various strategies have been determined.

The standard scheme for induction therapy has been three days of anthracycline (daunorubicin at least 60 mg/m2 or idarubicin 10-12 mg/m2) and 7 days of cytarabine continuous infusion of 100-200mg/m², a so called “3+7” regimen [58]. There has been no significant difference in complete remission or overall survival (OS) concerning the different anthracyclines used, however idarubicin seems to be superior in disease free survival (DFS) for patients not receiving an allogeneic stem cell transplantation (SCT) [162]. In patients younger than 60 years the CR rate is between 70-80% [26]. Certain modulations to the “3+7” scheme to improve remission rate haven been investigated. For example high dose cytarabine combined with daunorubicin has been studied in several clinical trials, however neither of them showed a higher remission rate, but increased toxicity was detected [36, 181]. The investigation of dose intensification for daunorubicin 90mg/m² combined with cytarabine showed improved CR

rates and longer OS [78]. The addition of a third cytotoxic agent like thioguanine, etoposide or fludarabine failed in general [58]. Other trials investigated the use of granulocyte stimulating factor (G-CSF) as a sensitizer for leukemic cell to increase the chemotherapy cytotoxicity, however due to diverse results on OS, it is not suggested to apply G-CSF outside of clinical trials [20, 58, 157]. Elderly (>60 years) patients usually if capable receive a reduced dosage of anthracycline. However older patients only achieve a CR in about 50% and an OS of just 12% [58]. This is mainly due to the adverse cytogenetics or reduced anthracycline sensitivity [28]. Patients that are not able to tolerate an intensive chemotherapy scheme should receive an investigational therapy, however if such a treatment is not available mild cytoreductive therapy is suggested.

Postremission therapy is more variable and therefore options different strategies like intensive conventional chemotherapy, prolonged maintenance treatment, high dose therapy followed by autologous or allogeneic SCT. High dose cytarabine (HiDAC) is the most accepted intensive chemotherapy, given at 3g/m² per 12h on days 1, 3, 5, for four monthly courses. HiDAC proved to be superior to intermediate- or standard-dose. The United Kingdom Medical Research Council (MRC) concluded after several years of clinical research that four courses are sufficient [27] and the supplementation of additional agents appears not to improve the outcome [170]. However, the favorable effect of HiDAC treatment seems to be limited to core binding factor (CBF) AML, cytogenetically normal (CN) AML including *NPM1* mutated AML without mutations in the *FMS-like tyrosine kinase 3 (FLT3)* gene (NPM1 mono) and *CEBPA* mutated AML [12, 202]. Maintenance therapy should not be generally administered outside of clinical trials except for APL, where a maintenance strategy has been approved [58]. Autologous SCT may be beneficial in specific subsets of AML, however there has been no evidence of an advantage for the outcome generally [58].

The postremission treatment with the lowest relapse rate, relying on myeloablative therapy and graft versus leukemia effect, is the allogeneic SCT. However, this procedure has also the highest therapy related mortality rate of about 20%-50%. Therefore patients with a favorable risk (Table2) constellation including CBF AML, *NPM1* mono and *CEBPA* mutated AML are not considered to undergo allogeneic SCT in first CR [46]. Clinical trials assigning allogeneic SCT

compared to alternative consolidation chemotherapies showed that allogeneic SCT improves OS significantly in patients with intermediate and high risk AML [58]. Nevertheless, the decision to perform an allogeneic SCT is not only based on the leukemia profile, but several factors are essential and must be considered including: matched donor status; age; comorbidity score, course of disease, and cytomegalovirus status of recipient and donor. Around 3% of the patients undergoing intensive induction therapy have refractory disease, which predicts poor outcome [201]. These patients should receive a salvage therapy within a clinical trial to achieve at least a partial remission (PR) followed, if eligible, by an allogeneic SCT.

Over the last couple years new genotype-specific therapeutic strategies have been evolved. These genotype-specific treatment approaches are currently under clinical investigation. In *FLT3*-ITD mutated AML several tyrosine kinase inhibitors such as midostaurin, lestaurinib or sorafenib as first generation inhibitors and quizartinib and crenolanib as second generation FLT3 inhibitors are applied in clinical trials [74, 90, 226]. Another tyrosine kinase inhibitor, dasatinib; originally developed for Philadelphia Chromosome positive CML treatment, showed promising results in a *KIT* mutated t(8;21) AML mouse model [227] and is therefore evaluated in ongoing clinical trials in CBF AML (e.g. NCT00850382: www.clinicaltrials.gov). Gemtuzumab ozogamicin, an anti-CD33 antibody linked with the cytostatic drug calicheamicin, is showing a revival after getting disposed in 2010 from the market for the treatment of older AML patients in combination with induction therapy. Two studies showed a significantly increased survival in elderly patients, however only in favorable and intermediate cytogenetic risk groups [29, 37]. This effect could not be confirmed with the combination of gemtuzumab ozogamicin and low dose cytarabine (LDAC), where it only improved response but not OS [30]. Another new therapeutical target are deregulated signaling pathways. The key enzyme for mitosis and cell cycle regulation, the polo-like kinase 1 (PLK1), represents such a new target. In a recently published clinical trial the polo like kinase inhibitor volasertib plus LDAC vs. LDAC alone provided a higher response rate, event free survival and OS in elderly patients not suitable for induction therapy [59]. Furthermore, epigenetic changes and their role in leukemogenesis are of growing importance in leukemia research and therefore it is not surprising that epigenetic changes have already been targeted in AML therapeutically. Two demethlyating substances

named azacytidine and decitabine have been approved for the treatment for AML and MDS. They will be discussed in detail in the chapter epigenetic therapy. In conclusion, it can be said even if the basis of anti-neoplastic therapy in AML may remain constant little steps forward are being made to more precisely detect subtypes for better patient adjusted therapy.

1.1.6 Prognosis and Risk stratification:

A number of patient related factors and biological characteristics reflecting the heterogeneity of AML influence the prognosis. For the patient related factors the patient's age is the most significant one, adversely affecting both achievement of CR and OS (Table3). Even after accounting for other risk factors like cytogenetics, molecular genetics, AML type (de novo, t-AML or s-AML) and performance status, older patients show a worse outcome than younger adults. This effect may rely primarily on different AML biology, concurrent co-morbidities, multidrug resistance protein positivity, and prior MDS [70]. However, calendric age should not decide alone, whether a patient is eligible for a potential curative, i.e. intensive, treatment approach. Therefore performance status and the application of a comorbidity score like the hematopoietic cell transplantation comorbidity index (HCTCI) are essential for the ultimate decision for treatment [58].

Tab.3: Impact of age on outcome in acute myeloid; in MRC acute myeloid leukemia trials (United Kingdom Medical Research Council) [211]

Age in years	5 year survival
0-14 years	63%
15-34 years	55%
35-44 years	46%
45-59 years	35%
60+	14%

The tumor burden of the AML reflected by the presence of clinical markers like hepatosplenomegaly, a high serum lactate dehydrogenase (LDH) level and a high WBC are associated with an adverse outcome even occurring in a cytogenetic favorable disease [129, 130, 173] Interestingly, a Canadian study showed that a high WBC with a cut-off value of $>30x10^9$/l is a significant harmful factor for death in induction therapy [103]. Other AML-related prognostic factors involve AML arising on preexisting hematological disorder like MDS or myeloproliferative syndrome (MPS), prior chemo- or radiotherapy related disease, as well as cytogenetic and molecular genetic aberrations found in leukemic cells.

The most essential prognostic factor for AML patients besides age is the karyotype of the malignant myeloid cells [83]. Aberrations of the karyotype are observed in about 50-60% of all AML patients. Based on cytogenetic and molecular genetic changes, patients are usually classified into four risk groups: favorable, intermediate I, II and adverse (Table4) [58].

Tab.4: Risk classification based on cytogenetic and molecular changes in AML [58] (AML=acute myeloid leukemia;BCR-ABL1= brealpoint cluster region Abelson murine leukemia viral oncogene 1; CBFB= core binding factor beta CEBPA= enhancer binding protein alpha; EVI1= ecotropic viral integration site-1; FLT3-ITD=FMS like tyrosine kinase 3- internal tandem duplication; MLL=mixed lineage leukemia, MYH11= myosin heavy polypeptide 11; NK= natural killer; NOS= not otherwise specified; NPM1= nucleophosmin, NUP214=nucleoporin 214; PML= promyelocyte leukemia ;RARA= retinoic acid receptor alpha; RPN1= ribophorin 1; RUNX1= runt related transcription factor; WHO= world health organization)

Genetic group	Subsets
Favorable	
	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD (normal karyotype) Mutated *CEBPA* (normal karyotype)
Intermediate-I	
	Mutated *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	
	t(9;11)(p22;q23); *MLLT3-MLL* Cytogenetic abnormalities not classified as favorable or adverse
Adverse	
	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* t(6;9)(p23;q34); *DEK-NUP214* t(v;11)(v;q23); *MLL* rearranged -5 or del(5q); -7; abnl(17p); complex karyotype

The favorable cytogenetic group includes patients with CBF AML with the translocation t(8;21), inv16 or t(16;16), as well as the APL associated aberration t(15;17). This group according to the Cancer and Leukemia Group B (CALGB) has a CR, relapse risk, and a five year OS of 88%; 54% and 55% [33]. In children and younger adults these favorable cytogenetics aberrations account for about 25% of all AMLs. Multivariable analyses provided that additional cytogenetic abnormalities, irrespective of complexity, occurring with favorable cytogenetics do not have an adverse impact on the patient's outcome [211]. But in inv(16) AML secondary abnormalities, especially +22, have been shown to be associated with improved outcome according to German AML Intergroup [201]. For this favorable group no allogeneic SCT is intended for patients achieving first CR.

In 10-20% of AML patients adverse cytogenetic changes will be diagnosed. Their CR, relapse risk and five year OS is 32%, 92% and 5%, respectively [33]. In this group monosomies of chromosomes 5 and/or 7 (-5/-7), deletions of 5q [del(5q)], 17p deletions (-17p), abnormalities of chromosome 3 [abn(3q)/ inv(3)/ t(3;3)], translocations involving 11q23 [other than t(9;11); see also Table4], t(6;9), t(9;22) or a complex karyotype are typically found [211]. A complex karyotype, found in 10%-12%, is defined as the occurrence of more than 3 chromosomal aberrations in the absence of CBF AML, t(9;11) and t(15;17) [58]. Patients diagnosed with adverse cytogenetic changes should receive, if eligible, an allogeneic SCT provided that they go into remission after induction therapy [46].

The most variability lies within the classification of the intermediate risk group cases. The MRC study group defines the intermediate group as entities not classified as favorable or adverse cytogenetics and AML with normal karyotype [105]. In contrast Byrd et al includes also AMLs with certain chromosomal changes like monosomy Y (-Y), t(9,11) and isolated trisomies like +8, +11, +13, +21 [33].The European LeukemiaNet (ELN), an international expert panel, distinguishes two subgroups for the intermediate risk group by separating cytogenetically normal karyotype with specific molecular genetic changes like mutation in the *NPM1* gene with or without concurrent *FLT3*-internal tandem duplication (*FLT3*-ITD) (Table4) [58]. As already mentioned above, due to the progress made in the detection of molecular abnormalities in leukemic cells, molecular genetics are more and more included in the risk stratification and

therefore influence the prognosis as well as the treatment strategy. So far mainly patients with a cytogenetically normal karyotype, about 40%-50%, rely on the identification of molecular genetic changes to improve the prognostic value. While this model suggest further improvements by incorporating novel poor prognostic markers such as sex combs like 1 (*ASXL1*), *RUNX1* and *TP53*, findings nevertheless need to be investigated within large prospective clinical trials before these markers can be moved forward into the clinic.

These days, only three somatically acquired mutations involving *NPM1*, *CEBPA* and *FLT3* are routinely applied in the clinical field, which affect also the treatment strategy [163]. NPM1 is a chaperone protein mostly prominent in the nucleoli, shuttling between nucleus and cytoplasm. It functions as a regulator and transporter for peri-ribosomal particles through the nuclear membrane. Additional to this it is involved in the regulation of the p53 tumor suppressor pathway [73]. However, due to mutations in exon 12 of the gene, NPM1 is mislocated persistently in the cytoplasm [73]. In about 50% of normal karyotype and 30% of all AML patients *NMP1* mutations are found, thereby making *NPM1* mutations the most affected molecular aberration found in leukemia cells [69, 202, 211]. In approximately 40% and 10-15% *NPM1* mutation is associated with *FLT3*-ITD and *FLT3*-tyrosine kinase domain (*FLT3*-TKD), respectively [163]. In CN-AML patients with a *NPM1* mutation without *FLT3*-ITD CR rate and OS are increased in younger as well as in elderly patients. This group of CN-AML may not be accounted for an allogeneic SCT in first CR [202, 203]. Relating to these findings patients with mutated *NPM1* without *FLT3*-ITD genetic aberrations have been allocated to the favorable risk group together with CBF AML [58]. AML with *NPM1* mutations are eligible for MRD monitoring [143].

FLT3 belongs to the class III receptor tyrosine kinase family and is expressed on hematopoietic progenitors. It plays a pivotal role in proliferation, survival and differentiation of hematopoietic progenitor cells [99]. *FLT3* mutations are detected in about 30% of all AML, thereby being the second most to *NPM1* [163]. There are two subsets of mutations one being more common, an in-frame internal tandem duplications, frequently found in exon 14 compromising the tyrosine rich juxtamembrane domain, which regulates the receptor's kinase activity [17], and mutations identified in the tyrosine kinase domain [241]. The former one is detected in approximately

20%, the latter in 10% of all AML cases [163]. The influence of *FLT3*-ITD on relapse risk and OS in normal karyotype AML is adverse compared to CN-AML without the mutation [202]. This decreased outcome is particularly seen in AML with a high *FLT3*-ITD allele ratio [171]. However the prognostic value of *FLT3*-TKD mutations remains controversial [58].

CEBPA, encoding a transcription factor, is essential for the regulation of myeloid differentiation. *CEBPA* mutations are mainly seen in CN- and 9q deletion AML [163]. There are two major mutations found in *CEBPA* one affecting the N-terminal region the other the C-terminal region. In about 70% N- and C-terminal mutations occur together, namely N-terminal mutations affecting one allele and C-terminal mutations the other allele [220]. *CEBPA* mutations have been associated with a favorable outcome like AML mutated with *NPM1* without *FLT3*-ITD [202]. However, it is important to mention that only double mutations, but not single *CEPBA* mutations, are associated with an improved outcome. Patients with a single *CEPBA* mutation seem to have a similar risk than *CEBPA* wild type AML [220].

The list of genetic aberrations potentially impairing the prognosis is increasing. Mutations in the isocitrate dehydrogenase gene *IDH1* and *IDH2*, occurring in approximately 30% in CN-AML, are associated with an adverse prognosis in younger and molecular low risk patients [180]. The impact of Wilms tumor gene (*WT1*) mutations detected in around 12% of normal karyotype AML seems to be controversial due to distinguished findings of two study groups [163]. A Chinese study recently published that *RUNX1* mutations found in 13 % worsen the CR rate and OS [219]. KIT, another receptor tyrosine kinase class III, mutations quite frequently found in CBF-AML with an incidence of 25-30% has been mostly associated in different study groups with an inferior outcome [179]. In AML with complex karyotype *TP53* alterations occur approximately in 70% of cases. It was shown that *TP53* alterations are significantly associated with a higher degree of genomic complexity as well as with a higher frequency of specific copy number alterations, especially aberrations associated with adverse outcome such as -5/5q-, -7/7q-. Additionally in a multivariable analysis *TP53* alteration was the most important prognostic factor [195]. Furthermore, subsets of molecular aberrations and their clinical influence are currently under investigation [58, 163, 211]. Gene expression (GEP) analysis showed that gene expression changes of certain genes including brain and acute leukemia

cytoplasmic (*BAALC)*, meningioma 1 (*MN1)*, ETS-related gene (*ERG)*, ecotropic viral integration site 1 (*EVI1)* have an adverse influence on the clinical course of AML patients [163].

1.2 Molecular-genetics:

Over the last thirty to forty years cytogenetics and the investigation of single candidate genes have identified recurrent somatic perturbations in myeloid malignancies. Nevertheless the molecular pathogenesis of acute myeloid leukemia has not yet been completely defined. There are clear identifications that specific genetic aberrations contribute to leukemogenesis [84]. The specific mutational events required for the clonal expansion are currently not well defined. Multiple data proclaim that a single mutation is not sufficient to cause the transformation from an early healthy myeloid cell into a malignant myeloid blast [60]. The commonly found AML translocations t(8;21), t(16;16)/inv(16) and t(15;17) impair myeloid differentiation, respectively but aren't sufficient to cause a leukemic phenotype mouse models [41, 88, 144]. However, 45% of AML cases have no cytogenetic aberrations and many cases lack recurrent structural changes even with high density comparative genomic hybridization and single nucleotide polymorphism (SNP) microarray-based analysis [24]. Therefore targeted sequencing investigations supplied several recurrent mutations that are not only pivotal for diagnostic and prognostic information, but that most likely contribute also to leukemogenesis (involved genes are *FLT3, NPM1, CEBPA, KIT, RUNX1,* neuroblastoma RAS *(NRAS), WT1*) [60].
An indication for the multi-step process necessary to achieve a malignant phenotype can be seen in leukemias arising from germline mutations in *RUNX1* and *CEBPA*. These mutations predispose for development an AML, if a second somatic mutations in hematopoietic progenitor cells occur [60]. Genetic and functional studies have provided that there are at least two classes of gene mutations needed that contribute to leukemic transformation. Class I comprises mutations affecting signal transduction pathways (Table5) leading to increased proliferation. The second class includes genes coding for transcription factors or transcriptional co-activation complexes (Table5) resulting in perturbed differentiation [61]. However, there has been evidence that there are additional leukemia disease mutations that cannot be determined by this previous two-hit model of myeloid pathogenesis. Moreover, in about 50%

of AML patients no ”classical” class I mutations can be detected and to date genetic studies were not able to identify additional class I mutations [208].

Even more importantly, recent data elucidated additional classes of mutations commonly found in AML. Of special interest are genes that encode proteins associated with DNA methylation and histone modification [208]. It has been provided that somatic mutations in epigenetic regulators are prevalent in myeloid malignancies and promote hematopoietic transformation [231]. The somatic mutations in epigenetic regulators occur concurrent with class I and class II mutations, however experimental studies have proclaimed that alterations of these epigenetic modifiers, eventually called class III genes, alone can lead to myeloid transformation [61]. Mutated genes encoding for an epigenetic modifying protein include ten eleven translocation (*TET2*), *IDH1/IDH2,* DNA methyltransferase 3A (*DNMT3A*), *ASXL1,* enhancer of zeste homolg 2 (*EZH2*)*,* BCL6-corepressor (*BCOR*) and PHD finger protein 6 (*PHF6)* (Table5) [43, 79, 89, 106, 150, 231]. Welch et al proclaimed that mutations in *NPM1*, *DNMT3A* and *IDH1* are more likely initiating mutations [231]. Furthermore *DNMT3A, TET2* and *IDH1* have been shown to promote self-renewal and block differentiation of HSC. Therefore the appearance of these mutations in HSC seem to lead to clonal expansion gathering in pre-leukemic stem cells [39, 124, 200].

Tab. 5: Potential gene mutation classes in acute myeloid leukemia [61]; (ASXL1=sex combs-like 1;BCOR= BCL-6 co-repressor; CBFB= core binding factor b; CEBPA= enhancer binding protein alpha; DNMT3a= DNA methyltransferase; EZH2= enhancer of zeste homolog 2; FLT3= FMS like tyrosine kinase 3; IDH1/IDH2= isocitrate dehydrogenase; JAK2= janus kinase 2; KRAS= Kirsten rat sarcoma viral oncogene; MLL= mixed lineage leukemia, NPM1= nucleophosmin; NRAS= neuroblastoma RAS viral oncogene homolog; PHF6= PHD finger protein 6; PTPN11= protein tyrosine phosphatase; RUNX1= runt related transcription factor, RARA= retinoic acid receptor alpha)

Class I	Class II	Class III
Signal Transduction	Differentiation	Epigenetic regulation
FLT3	*RUNX1*	*TET2*
KIT	*CBFB*	*IDH1, IDH2*
NRAS, KRAS	*CEBPA*	*DNMT3A*
JAK2	*NPM1*	*ASXL1*
PTPN11	*PU1*	*EZH2*
	MLL	*BCOR*
	RARA	*PHF6*

Besides aberrant functional epigenetic regulators due to somatic mutations recent investigations were able to show that following epigenetic modifiers are perturbed due to recurrent translocations: lysergic acid diethylamide 1 (*LSD1*), nuclear receptor-binding SET domain protein 1 (*NSD1*), and MLL [51, 111, 142]. Most of the epigenetic aberrations worsen the outcome and seem to mainly occur in elderly patients [61].

The most studied and best understood epigenetic aberration and its role in leukemogenesis is the *MLL* translocation [142]. The *MLL* gene is located on chromosome 11 and is a frequent partner for recurrent translocations in AML. It functions as histone 3 lysine 4 (H3K4) methyltransferase possessing a DNA binding domain and positively regulating gene expression including homeobox (*HOX)* genes [142]. Inter-chromosomal translocations affecting the N-terminus of MLL can create fusion proteins with the C-terminal end of over 50 different fusion partners, thereby resulting in a loss of the H3K4 methyltransferase domain [142]. In mouse models, the MLL chimeric fusion proteins can cause a transformation of hematopoietic progenitors into leukemia stem cells [141].

1.2.1 Epigenetics:

The diversity within our population is not explainable with the classic genetic model and neither is the complex cancer biology fully comprehensible just by somatic mutations or genome alterations. Epigenetic changes, termed as heritable changes in gene expression that are not caused by any alteration in the DNA sequence [116], may have filled the hole. Epigenetic driven processes are essential in all aspects of cellular biology including growth, differentiation, and apoptosis and therefore also in initiating a distinct cellular phenotype [11]. However over the last decades epigenetics has been of special interest due to its role in the development of malignancy [68, 127]. In contrast to DNA mutations, epigenetic changes are reversible and have been therefore the subject of multiple pharmacological studies for new cancer therapies [119, 192]. The entire epigenetic information, called the epigenome, adjusts during cellular development and remains preserved during cellular replication and sometimes is even able to be inherited through the germ line [42]. In addition, the importance of epigenetics is highlighted by the fact that genome-wide sequences are identical in actually all healthy cell and tissue types of an individual, and the epigenome plays a crucial role in cell fate decisions [63]. In cancer these epigenetic patterns are perturbed in relation to the healthy tissue [66]. Interestingly Costello et al clarified that these DNA methylation patterns found in malignancies are non-random and each tumor type defines its own distinct characteristics [47]. So far three primary characteristics have been found that compromise the epigenetic information: DNA methylation and histone modification. DNA methylation is the best understood epigenetic process [114]. Within this thesis the main focus will be based on DNA methylation due to being the biological target of decitabine.

DNA methylation is the best studied and understood epigenetic factor [10]. At the 5-position of the cytosine ring the DNA is methylated by DNA methyltransferases (DNMTs), that either maintain methylation during replication by copying of the parental-strand methylation pattern onto the progeny DNA strand (DNMT1), or that are involved in the de novo methylation process of previously unmethylated DNA (DNMT3A and DNMT3B) [10]. The DNA methylation occurs during the DNA replication. The DNMTs create an intermediate complex with the cytosine ring and incorporate S-adenosyl-L-methionine (Ado-Met). This leads to a transfer of a methyl group from the Ado-Met to the C5 of the cytosine residue. The methylated cytosines

primarily occur in the context of CpG dinucleotides (cytosine precede to guanosine and bridged by a phosphate group). The CpG dinucleotides may be poorly represented over the genome, however they are not randomly distributed. CpG sites of extraordinary rich GC appearance, so called CpG islands (CGIs), are located in approximately 50% in the 5' ends of the regulatory regions of genes [217]. Under physiological circumstances these CGIs are unmethylated in cells; however there are subgroups of promoter CGIs that are normally methylated in tissue-specific patterns of gene expression like X chromosome inactivation [212]. How significant DNA methylation is for proper cell function can be strikingly seen in DNMT-knockout mouse model leading to embryonic lethality [175]. DNA hypomethylation of the CGIs commonly seen in healthy tissues usually are permissive for gene expression, however DNA hypermethylation seen in cancer cells results in silenced gene transcription due to recruiting of transcription repressor proteins as well as directly blocking transcription factors binding [86]. Nevertheless, the global genome methylation status of cancer cells compared to their healthy counterpart is decreased and the hypermethylation generally presented in CGIs is rather a non-random aberrant distribution and likely an essential step in the carcinogenesis [75]. The global loss of methylation is causing chromosomal instability and loss of imprinting [68]. In addition to these findings hypermethylation of regulatory gene regions is leading to a decrease expression of tumor suppressor genes favoring the occurrence of cancer [67, 125].

Histone proteins, four in number (H2A, H2B, H3 and H4), package the DNA into so called nucleosome units, and the repeated sequence of nucleosomes composes the chromatin. The amino-terminal histone tails protrude out of the nucleosome and are a target structure of posttranslational modifications including acetylation, methylation and phosphorylation. These histone modifications are influencing how tightly or loosely the chromatin is compacted. This mechanism has direct effects on cellular processes like gene transcription, DNA repair, DNA replication and chromosome organization [125].

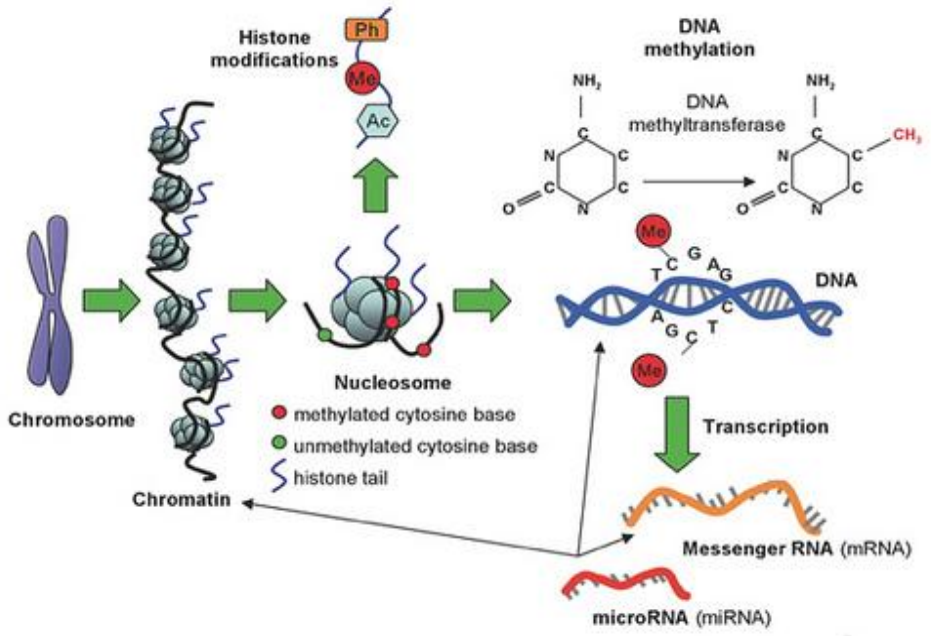

Fig.2: Epigenetic changes of the DNA [101] (Ac= acetyl; Me= methyl, Ph= phosphorylation; A= adenine, C= cytosine; G= guanine; T= thymine)

Histone acetyltransferases (HAT) are in charge for acetylation of lysine residues of H3 and H4, which are mainly associated with transcriptional activation. The histone methylation consequences rely on the amino acid type and its position in the histone tail and therefore can either lead to repression or activation of gene transcription. The methylation of H3K4 for example is an established epigenetic characteristic for activation. In contrast, trimethylation of H3K27 acts as a repressor [68, 185, 245]. A good illustrated gene example for the above mentioned processes is the tumor suppressor gene *p21*, which is silenced due to histone hypoacetylation and hypermethylation of H3 and H4 even with the lack of CGIs promoter methylation [82].

Interestingly these two epigenetic mechanisms DNA methylation and histone modifications are strongly interdependent. This interaction was detected by the discovery of demethylation capability of histone deacetylase inhibitors, as well as the association between histone methyltransferases with DNMTs [185].

While recently significant efforts were made to elucidate the mechanisms of leukemogenesis, the increased knowledge of epigenetic changes was essential and is gaining momentum, however we are still far away of fully understanding, how the epigenetic alterations contribute to myeloid transformation [41, 164, 185]. In the late 1980s the first findings of abnormal DNA methylation in the 5' regulatory region of the calcitonin gene in leukemia was described [8]. Subsequent studies were able to prove that aberrant epigenetics is a hallmark of leukemia [41]. Genome wide restriction landmark genomic scanning provided that up to 8.3% of CGIs are altered due to elevated DNA methylation; taking this into relation with absolute numbers, about 2000 CGIs are aberrantly methylated or over 2000 genes are repressed [197, 221]. In a next generation genome wide DNA methylation analysis aberrantly methylated CGIs could be determined in myeloid cell lines. Interestingly, genes associated in transcriptional regulation were significantly over represented within the hypermethylation patterns. Furthermore this analysis showed that leukemia or normal monocyte-derived microarray expression data sets lacked more than 50% of aberrantly methylated genes [95]. Given the existence of cancer specific methylation patterns, AML can be divided in biological defined subtypes based on the methylation signatures [80]. Several study groups identified that the changes of the methylation characteristic in malignant myeloid blasts predicts survival in AML patients and supports to include DNA methylation patterns prospectively to the variables for prognosis and risk stratification [23, 52, 80]. Cytogenetic AML subtypes including inv16, t(8;21), t(15;17) and 11q23 feature each an unique and distinct methylation signature. Similarly, *NPM1* and *CEBPA* double mutations each display a specific epigenetic cluster however for FLT3 aberrations no association was seen with distinct epigenetic signatures [4, 80].

Today many genes with aberrant epigenetic regulation have been identified just to mention a few including *p15, p16,* retinoblastoma-interacting zinc-finger protein 1 *(RIZ1),* brain and muscle ARNT-Like 1 *(BMAL1),* exostosis type 1 *(EXT1),* inhibitor of DNA binding 4 *(ID4),* cadherin 1*(CDH1)* and genes involved in the WNT as well as in the mitogen activated protein kinase (MAPK) signaling pathway though their prognostic influence remains largely unknown [44, 68, 114]. The question what event is the driving force remains unclear, either epigenetic aberrations occur as a consequence of genetic mutations/translocations or the epigenetic programming precedes the genetic mutation/translocation by causing chromatin instability

[164]. An example for the former was presented by Figueroa et al by showing that mutations of the *IDH1/IDH2* genes lead to a hypermethylation phenotype and inhibit *TET2* an enzyme functional in the demethylation process [79]. In APLs the onco-fusion protein PML-RARA seems to be associated with the recruitment of HDACs and DNMTs for hypermethylating promoters compromising a retinoic acid response element generating gene silencing [56]. In t(9;11) leukemia resulting in the onco-fusion protein MLL-AF9 the chimeric protein loses its H3K4 methyltransferase activity thereby causing gene expression changes and aberrant DNA methylation [142]. Interestingly, the majority of MLL fusion partners seem to be associated with the DOT1L methyltransferase that increases transcription by methylation of histone H3 lysine residue 79 (H3K79) [174]. It could be shown that induced over-expression of MLL-AF9 drives aberrant DNA methylation signatures like the ones seen in t(9;11) AML samples. However, the AML-ETO1 and CBFB-MYH11 fusion proteins emerging out of t(8;21) and inv(16) alone are not capable of recapitulating the methylation signature observed in the respective primary AML samples [4]. Therefore it can be suggested that for these AMLs the epigenetic changes might have occurred earlier thereby being the primary events. This hypothesis is underscored by the detection of mutations in epigenetic driver genes like *DNMT3A, IDH1* and *TET2* in pre-leukemic stem cells [40].

1.2.2 Epigenetic therapy in malignancies:

Due to the evolving role of epigenetic aberrations in the tumorigenesis and their high prognostic impact generating new therapy aspects by targeting these molecular mechanisms has been part of ongoing research and some of them were already included in the clinical field. Despite genetic mutations, which are irreversible, epigenetic changes can be reversed by pharmacological inhibitors. The best known drug that is able to reverse epigenetic changes is ATRA in the therapy of APL [41].

The two main classes of epigenetic active drugs are DNA methyltransferase inhibitors and histone acetylase inhibitors (HDACI), but both classes show substantial limitations in their epigenetic specificity [192]. In the group of DNA methylation inhibitors nucleoside and non-nucleoside analogues have been under investigation, however so far only the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved nucleoside

analogues named decitabine (DAC) and azacytidine (AZA) are approved for the daily practice for AML and MDS patients. These drugs consist out of a modified cytosine ring that is attached either to a ribose or deoxyribose. After the incorporation into DNA or RNA, subsequently the DNA methylation is supposed to be blocked. This mechanism does not lead to immediate cell death, because the cells must be allowed to proliferate and re-express genes that have been silenced due to methylation. Then, the reactivation of genes involving apoptosis genes and cell cycle regulators is a volitional effect, which subsequently generates cell death, cell cycle arrest and cell differentiation [245]. However, since the epigenetic aberrations in malignant cells are reversible this might imply that the drug effect is transient leading to recurrence of the aberrant patterns after treatment. Early responses are rarely seen after one cycle and need time to improve [209]. Interestingly it seems to be quite common that patients that show no evidence of therapeutic response for 2-3 months go into complete remission if therapy is continued [159, 176]. Besides these two drugs, several other drugs have been applied in early clinical trials or on an experimental basis, but none of them has been accounted for practical clinical use so far. In the group of non-nucleoside analogues some have progressed into clinical trials and may evolve in the future clinical use. These small-molecule inhibitors differ from DAC or AZA by binding directly to the catalytic region of the enzyme without the need of DNA incorporation [18]. Besides hematological malignancies DNMT inhibitors have also been under investigation in clinical trials for solid tumor therapy like melanoma, breast, renal cell, colon and bladder cancer [245]. However in solid tumors it seems that only the combination of DNMT inhibitors and HDACI result in a sufficient tumor cell growth inhibition [196].

So far, two HDACIs have been FDA approved, vorinostat (VST) and romidepsin (RDS), both for cutaneous T-cell lymphoma (CTCL) [192]. The biological effect of HDACIs relies on the inhibition of the histone deacetlyase enzyme resulting in the accumulation of acetylated histones, which leads to a re-activation of cellular processes that have been abrogated in malignant cells [245]. This mechanism affects various genes associated with cell cycle regulation, apoptosis and angiogenesis. However, the exact mechanism how these drugs provide anti-tumor activity has not been elucidated yet. A potential mechanism includes the re-activation of tumor suppressor genes and repression of oncogenes, as well as the enhanced transcription of intrinsic apoptosis pathways and providing genomic instability [41]. VST, a hydroxamic acid approved for the

treatment for CTCL, features also some activity in AML. Based on a phase I trial that demonstrated VST has limited but single agent activity in advanced AML and MDS [92], a recently published phase II trial combining VST with idarubicin and cytarabine showed that this combination is well tolerated and active in AML [94]. Besides VST panobinostat (PBS) another hydroxamic acid represents also a highly anticipated HDACI currently under clinical investigation in AML trials, as it shows synergistic effects with doxorubicin and histone methyltransferase inhibitors in human AML cell lines [81, 161]. These results led to the start of a new trial comparing AML standard therapy versus standard therapy plus VST. For RDS a bicyclic depsipeptide antibiotic isolated from the bacterium chromobacterium violaceum with antineoplastic activity [182], there is little clinical experience about its anti-leukemic function in AML [137]. The short-chain fatty acid valproic acid, an established anti-epileptic drug, but also one of the first known HDACIs, is used as an additional drug in new experimental AML therapy strategies. In combination with ATRA in elderly AML patients valproic acid presents promising results [189]. DAC combined with valproic acid showed good response rates, however dose-limiting encephalopathy occurred in elderly AML patients [13]. In a trial treating AML/MDS patients with AZA plus valproic acid without ATRA suggested that valproic acid provides substantial additional benefit [145]. In conclusion, epigenetic drugs, whether DNMT inhibitors or HDACIs, can be used as single drugs or as part of a combination with classic chemotherapy and they provide favorable results.

Decitabine:

Synthesized in 1964 by Sorm and colleagues as a standard cytostatic drug, the anti-leukemic effect has been identified in 1968, and thus decitabine has been known now for almost 50 years [213]. Nevertheless intensive investigation on its therapeutic value in hematologic malignancies is still part of current leukemia research. DAC is a deoxyderivative of the pyrimidine analog 5-azacytidine (azacytidine) with the chemical term 5-aza-2-deoxycytidine.

The clinical interest for DAC started, when in-vivo studies provided evidence that DAC may be more potent than cytarabine in mice and that it shows terminal differentiation potential in murine embryonic as well as in primary leukemic myeloid cells [126, 169, 183]. In the early days of clinical application of DAC in the 1980s it was used as a standard antileukemic agent,

inducing DNA synthesis arrest and cytotoxicity emerged by DNA adducts, with a maximum tolerated dose of 1500 to 2500 mg/m² [49, 121]. However these dosages were associated with prolonged myelosuppression. Due to progress in understanding the cellular biology of epigenetics in myeloid malignancies and the biological action of DAC at lower doses, DAC was reevaluated in 1/20th of its former high dose applications [121, 126]. In lower doses DAC intervenes in the epigenetic regulation of genes by inhibiting the DNA methyltransferase leading to gene expression changes that promote differentiation, decreased proliferation and apoptosis [35]. However, clinical findings clearly indicate that the clinical response of hypomethylating agents cannot exclusively be accounted to the epigenetic gene alterations [93].

The DNA hypomethylating feature is restricted to cytosine analogs with 5' modifications of the ring like DAC or azacytidine [119]. The patterns of response from DAC or AZA are quite different compared to traditional cytotoxic therapy. In contrast to chemotherapy from which a rapid response is expected, response to hypomethylating agents are barely seen after on cycle and rather improve over time [76, 209]. Therefore, it is interesting to see that DNA methylation changes after DAC can already be found after a median of 6 days [45]. For patients receiving hypomethylating therapy it is not uncommon to achieve a CR even though there was no evidence of therapeutic efficacy for the first three months [176]. Because of this different pharmacological effect the regular side effects seen in traditional chemotherapy (mucositis, vomiting, nausea, organic toxicity etc.) are quite rare in DAC or AZA with side effects mainly concerning bone marrow function [188]. In May 2006 DAC received its approval from the FDA for the treatment of MDS, chronic myelomonocytic leukemia (CMML) and for the AML in September 2012 by the EMA.

DAC is administered as a prodrug that is depending on intracellular metabolically activation. The cellular uptake of the prodrug relies on human equilibrative nucleoside transporter hENT1 and hENT2 followed by activation on a phosphor-kinase deoxycytidine kinase (dCK) dependent manner into 5-aza-2`-deoxycytidine-5´triphosphate [49, 170]. The active metabolite will be incorporated by the DNA polymerase into DNA. Subsequently DAC builds a covalent complex with the DNA methyltransferase DNMT1 causing an irreversible blockage of the enzyme's

activity leading to newly hypomethylated synthesized DNA strands [199, 236]. In addition it has been suggested that DNMT1 is proteasomally degraded following DAC treatment [97]. Cytidine-deaminase found in the human liver, spleen, granulocytes, intestinal epithelium and plasma is responsible for inactivation of DAC through deamination. Due to high levels of cytidine-deaminase in the plasma, the plasma half-life time of DAC is only approximately 35 minutes [49, 121]. Nevertheless, the exact route of elimination and metabolic fate of decitabine is not fully known in humans. Because of the ability to pass the blood-brain barrier, the central nervous system (CNS) concentration of DACs achieves about 27%-58% of the plasma level after continuous infusion [38]. In a pharmacokinetic study of 15mg/m² DAC intravenously administered over 3 hours, every 8 hours, on 3 consecutive days repeated every 6 weeks it was shown that DAC does not accumulate systemically and that it is pharmacokinetic remains unaltered from cycle to cycle [34]. Compared to azacytidine DAC provides a more potent activity in inhibiting methylation at equimolar concentrations. This may be due to the primary incorporation of azacytidine into RNA and much lesser extent into DNA in contrast to DAC [115].

1.3 Gene expression profiling (GEP):

GEP in hematological malignancies:

Despite recent progress, the daily used diagnostic procedures to determine the specific type AML like bone marrow morphology, FACS analysis, cyto- and molecular-genetics are not sufficient enough to fully explain the heterogeneity within the AML. This is especially relevant for AMLs that appear to be cytogenetically and genetically normal. However this challenge does not only concern AML, but also other malignancies like breast cancer, lung cancer or lymphomas, which face the same questions if it comes down to explain the heterogeneity within the disease. Over the last 15 years the technology of genome-wide DNA microarray based gene expression analysis has evolved, which helps for a better understanding of biology of cancer especially within already defined subgroups [239]. In a landmark study in the late 1990s Golub et al. were the first who demonstrated the profitableness of GEP in hematologic malignancies by discriminating AML samples from ALL based on the distinguished GEP and a subsequently unsupervised analysis [100]. Beyond that, the study gained in importance due to providing three potential applications of GEP, namely class discovery, class prediction and class comparison [62, 239]. Class discovery is defined as the identification of new subgroups within the malignancies, whereas class prediction relies on already determined subgroups that are predicted based on gene expression data. The identification of genes deregulated between certain subgroups, class comparison, opens new biological insights into carcinogenesis [62, 239].

Based on the early study by Golub et al further investigational studies evolved over the last several years. Hence today the correlation of gene expression with certain molecular aberrations and the clinical outcome allows to characterize the disease in more detail. In addition, important cellular pathways as well as new clinical significant subgroups were able to be identified based on their GEP [62, 239]. In the entity of diffuse large B-cell lymphoma (DLBCL) two new prognostic relevant subgroups distinguished by the cell of origin were found and interestingly these two subgroups were also significantly different in their clinical outcome [3]. This analysis resulted in the clinical subclassification for DLBLC. Similarly, based on GEP in

360 pediatric ALL patients it was possible to identify distinct molecular subtypes of prognostic relevance, including T-ALL, E2A-PBX1, BCR-ABL, TEL-AML1, ALL with MLL rearrangement and hyperdiploid ALL with >50 chromosomes [244]. MLL rearrangement ALL contains a unique GEP including high expression of FLT3 [5], which is today used as a therapeutic target if mutated [247].

GEP in AML:

Based on gene expression analysis several groups were able to classify distinct cytogenetics AML subgroups including t(15;17), t(8;21), inv(16) and t(11q23) [21, 205, 222]. In addition distinct GEP were also determined correlating with molecular aberrations like *FLT3*-ITD, *NOTCH1*, *CEBPA* and *NPM1* [172, 222, 225, 237]. As mentioned above the GEP has the potential to uncover new subgroups within several types of cancer like DLBCL, breast cancer or ALL [3, 244]. Therefore several studies applied the GEP method for AML molecular investigations over the last years. Especially in normal karyotype AML are these analyses important due to the lack of known cytogenetic, molecular aberrations and their heterogeneity in clinical outcome. Two independent studies illustrated new molecular subtypes of AML including those with normal karyotype involving subgroups of different outcome [21, 222]. In an analysis of 116 AML cases Bullinger et al identified by an unsupervised analysis two subgroups with a normal karyotype and assumed that these subgroups may be clinically relevant and indicated an association with *FLT3*-ITD, FAB-M1 and M2 for group I whereas FAB-M4 and M5 were more common in group II [21]. Furthermore, high expression of genes like globin transcription factor 2 (*GATA2)*, *DNMT3A/DNMT3B* and for a lesser extent *NOTCH1* was characteristic for group I. In the survival analysis group I had a significantly poorer outcome that group II, underscoring a potential clinical influence of an aberrant methylation status due to high expression of methylation regulating genes [21]. Based on their findings they generated an optimal 133-gene clinical outcome predictor, applied on the normal karyotype subgroups it predicted a significant poor and good outcome, respectively [21].In a cohort of 285 cases of AMLs similar results were seen in a GEP experiment recognizing 16 subgroups by an unsupervised cluster analysis [222].

Subsequently, it was reported that within core binding factor AMLs GEP identified distinct subgroups that may explain the diversity in the clinical outcome by this group generally associated with a good prognosis [22]. In an unsupervised hierarchical cluster analysis they demonstrated a subgroup of CBF AMLs featuring a shorter survival. The evidence that GEP is able to discover a biologically significant subtype has been proven by a specific leukemia distinguished by epigenetic silencing of *CEBPA* and a myeloid/T-lymphoid phenotype [237, 238]. In this report it was shown that these leukemias were characterized to have a similar gene expression compared to AML with *CEBPA* mutations, however no such mutations were detected. Interestingly, in subsequent assays it was postulated that this phenomenon was due to epigenetic silencing of *CEBPA* and the pathway analysis adduced that the leukemias carried both myeloid and T-lymphoid characteristics [237]. This study underlines the benefit of the practicability of biologic pathway analysis to clinical GEP data.

1.4 Aim of this study:

The AML is a quite heterogeneous disease, both clinically and biologically. Epigenetic therapy is just at its early steps but has already become an adequate option for elderly patients as a treatment strategy. However, due to the lack of reliable clinical predictors for DAC therapy it is still uncertain who does actually benefit from this treatment. Therefore, the molecular diversity within the disease explaining the different response must be better understood to predict treatment response. With that regard GEP analysis has gained of importance and is pivotal tool for cellular biology studies and helps these findings to translate into the clinical practice.

In the following analysis we will investigate, whether it is possible to predict patients who respond to DAC therapy based on GEP in diagnostic AML samples. Furthermore, this investigation may help for a better understanding of the anti-leukemic effect of DAC, as well as to find out what is the leading pathomechanism behind treatment resistance, and based on these findings we might be able to establish clinically relevant predictors. Therefore, providing new molecular insight into the epigenetic therapy might further improve the value of transcriptomic based predictors and lead the way to improved patient management.

2 Material and Methods

2.1 Patients and treatment strategy:

All 46 AML patient samples were provided by the biobank of the German and Austrian AML Study Group (AMLSG). The informed consent for treatment, cryopreservation of samples and experimental analyses executed in the accordance with the Declaration of Helsinki and with institutional review board approval from all participating centers was signed by all patients. All patients were treated within the AMLSG multicenter trial: "Phase II study of low-dose intravenous decitabine in patients aged > 60 years with acute myeloid leukaemia who are not eligible for standard induction chemotherapy", registered as DRKS00000069 in the German Clinical Trial Registry.

Patients aged 61 years or older with previously untreated de novo or secondary AML following MDS who were not fit for intensive induction therapy were eligible. The diagnosis of AML or sAML was based on the FAB classification. The cytogenetic associated risk group stratification was based on the WHO classifications of 2008. All cytogenetic analyses were executed in the central AML laboratory of the Internal Medicine Department III of the University Hospital Ulm using conventional chromosome banding.

Patients were included with a Eastern Cooperative Oncology Group (ECOG) performance status between 0 and2, total bilirubin concentration less than twice the upper level of normal, serum creatinine less than 1.5 mg/dl except leukemia related and provided a signed informed consent was achieved. Previous treatment with hydroxyurea to decrease the white cell blood count was accepted. The exclusion criteria involved APL, cytokine treatment within the last 4 weeks, an additional existing malignancy not in remission, New York Heart Association (NYHA) grade IV heart failure, uncontrolled active infection, known allergy to imidazoles and psychiatric disorder interrupting therapy.

The study treatment comprised three times daily intravenously infusion of DAC in doses of 15mg/m^2 over three hours on three consecutive days (total dose 135mg/m^2) repeated every 6 weeks. If the response was anti-leukemic effect (ALE) or stable disease (SD) (for definition see

below) after the first cycle 1, ATRA was added from day 4 in the second cycle for a total of 28 days.

About three years after the study initiation a protocol amendment led to a change of the treatment strategy in the first therapy cycle for patients with a WBC greater than $20x10^9$/L. In detail, in these cases the DAC administration of days 1-3 was repeated after five days on day 8-10 of cycle 1, thereby leading to a total dose of 270mg/m^2. If the application of 4 cycles was successful, patients achieving CR, PR, or an ALE were eligible for maintenance therapy with DAC at 20mg/m^2 given for 1 hour on 3 consecutive days, repeated every 6-8 weeks. Response assessments were performed after cycles 1, 2, 3 and 4 by bone marrow aspiration. The response criteria are listed below in table 6.

Tab.6: Definition of response criteria in the clinical study (ALE=anti leukemia effect; CR=complete remission; PD= progressive disease; PR= partial remission; SD= stable disease; WBC= white blood cells)

Response	Definiton
CR	blast count <5%, platelets >100x10^9/L, WBC >1,5x10^9/L, no extramedullary leukemia
PR	blast count 5-25%, platelets >100x10^9/L, WBC >1,5x10^9/L or blasts <5%, platelets <100x10^9/L, WBC <1,5x10^9/L
ALE	>25% relative blasts reduction
SD	absence of CR, PR, ALE or PD
PD	>25% relative blasts increase

Patients who died from any cause within 6 weeks after therapy initiation of DAC were classified as early death [159].

Tab.7: Characteristics of patients treated with DAC (DNMT3A= DNA methyltransferase; FAB= French-American British; NPM1= nucleophosmin)

Characteristics		n=46
Age – n (%)		
Median (range)		**72 (61-85)**
	>75	**13 (30)**
	<75	**31 (70)**
	Missing	**2**
Gender - n (%)		
	male	**29 (64)**
	female	**16 (36)**
	missing	**1**
Performance status – n (%)		
	0	**5 (11)**
	I	**31 (71)**
	II	**8 (18.)**
	III	**0 (0)**
	Missing	**2**
FAB subtype – n (%)		
	M0	**2 (5)**
	M1	**20 (50)**
	M2	**13 (33)**
	M3	**0 (0)**
	M4	**3 (8)**
	M5	**0 (0)**
	M6	**0 (0)**
	M7	**2 (5)**
	Missing	**6**
Cytogenetic risk group – n. (%)		
	Low-risk	**0 (0)**
	Intermediate	**23 (62)**
	High-risk	**14 (38)**
	Missing	**9**
White blood cell counts (G/l)		
	median (range)	**3.4 (0.7- 186.4)**

<5,0 – n. (%)	**25 (55)**
5,0 – 19,9	**14 (32)**
20,0 – 49,9	**5 (12)**
> 50,0	**1 (2)**
Missing	**1**
Platelets (G/l)	
Median (range)	**52 (11- 474)**
< 50 – n (%)	**22 (48)**
> 50	**24 (52)**
Missing	**1**
Hemoglobin (g/dl)	
Median (range)	**8.8 (5.7- 12.6)**
< 10 – n(%)	**33 (73)**
> 10	**12 (27)**
Missing	**1**
Peripheral blood blasts (%)	
Median (range)	**11 (0- 92)**
< 10 – n (%)	**21 (49)**
10 – 30	**11 (25.5)**
> 30	**11 (25.5)**
Missing	**3**
Bone marrow blasts (%)	
Median (range)	**68 (0- 96)**
< 30 – n (%)	**3 (8)**
30- 49.9	**13 (33)**
> 50	**23 (59)**
Missing	**7**
Serum lactate dehydrogenase (U/L)	
Median (range)	**249 (92- 1720)**
< 300- n (%)	**29 (64)**
> 300	**16 (36)**
Missing	**1**
***DNMT3A* mutation – n. (%)**	
No	**37 (86)**
Yes	**6 (14)**

	Missing	**3**
***NPM1* mutation-n.(%)**		
	No	**30 (83)**
	Yes	**5 (17)**
	Missing	**1**

2.2 Isolation and storage of the AML patients sample:

The leukemic cells of the patient samples were isolated from the heparin peripheral blood and / or bone marrow by density gradient centrifugation using BiocollSeparating Solution according to the our routine SOPs (standard operating procedures) “Probenannahme-Bearbeitung, Lagerung und Dokumentation im Eingangslabor” (chapter 11). In brief, the peripheral blood mononuclear cells (PBMCs) or mononuclear cells from the bone marrow were isolated from the interface layer and washed with 1x phosphate buffered saline (PBS). The collected cells were stored at -80°C for RNA/DNA harvesting or cell lysis, or they were viably frozen in medium containing 10% DMSO (dimethyl sulfoxide) and stored in liquid nitrogen for subsequent experiments.

2.3 RNA-Isolation:

Before the gene-expression analysis and qRT-PCR could be executed isolation of the RNA from the patient samples was required. RNA was segregated from patient samples via Trizol reagent (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA). For this procedure cells had to be lysed before and homogenized in 1ml Trizol reagent per sample by recurrent passing through a syringe and incubated at room temperature for 5 min to guarantee a total dissociation of nucleoprotein complexes. After 200 µl Trizol was added to each sample, the tube had to be gently shaken by hand for 15s followed by further 3 min of incubation at room temperature. The next step was to separate the different phases. This was achieved through sample centrifugation at 4°C and 12’000g for 15 min. The RNA ends up as the aqueous phase (on top) with an average volume of about 600 µl. The RNA/aqueous phase was carefully removed and put into a new tube (RNase-free 1.5ml safe-lock, Eppendorf). The remaining

organic phase was stored at -80°C, for eventual subsequent experiments. The RNA was precipitated by adding 500 µl isopropyl alcohol to RNA/aqueous phase and incubation at room temperature for 10 min followed by centrifugation at 4°C and 12'000g for another 10 min. The supernatant was removed and 1 ml 75% ethanol was added to the RNA pellet followed by vortexing the sample and centrifugation at 4°C and 7'500g for 5 min. Subsequently the supernatant was removed again, and the RNA pellet was air-dried for about 4 min. The dried RNA pellet was then dissolved in 15-100 µl RNase-free water (Ambion) through recurrent pipetting and incubation at 60°C for 10 min. To evaluate the RNA concentration a Nanodrop photometer was used. The RNA quality was analyzed by agarose gel electrophoresis. For this 500 ng RNA per sample, 2 µl of 10x blue juice gel loading buffer and nuclease free water for a required volume of 10 µl were put on a 1.5% agarose gel (SeakemLELonza; TAE buffer) containing 0.5µl/ml ethidium bromide. The separation was performed with 110V amperage for 1-1.5 hours. Then the gel was visualized to characterize the RNA banding under UV-light (GeneSnap Software) and documented (GBox, Syngene). The RNA samples were stored at -80°C.

2.4 Gene expression profiling:

For the gene expressions profiling the isolated RNA was used. For this procedure the Affymetrix GeneChip 3'IVT Express KIT assay was executed for RNA target preparation for microarray expression analysis.

An overview for the single steps is listed and showed graphically below:

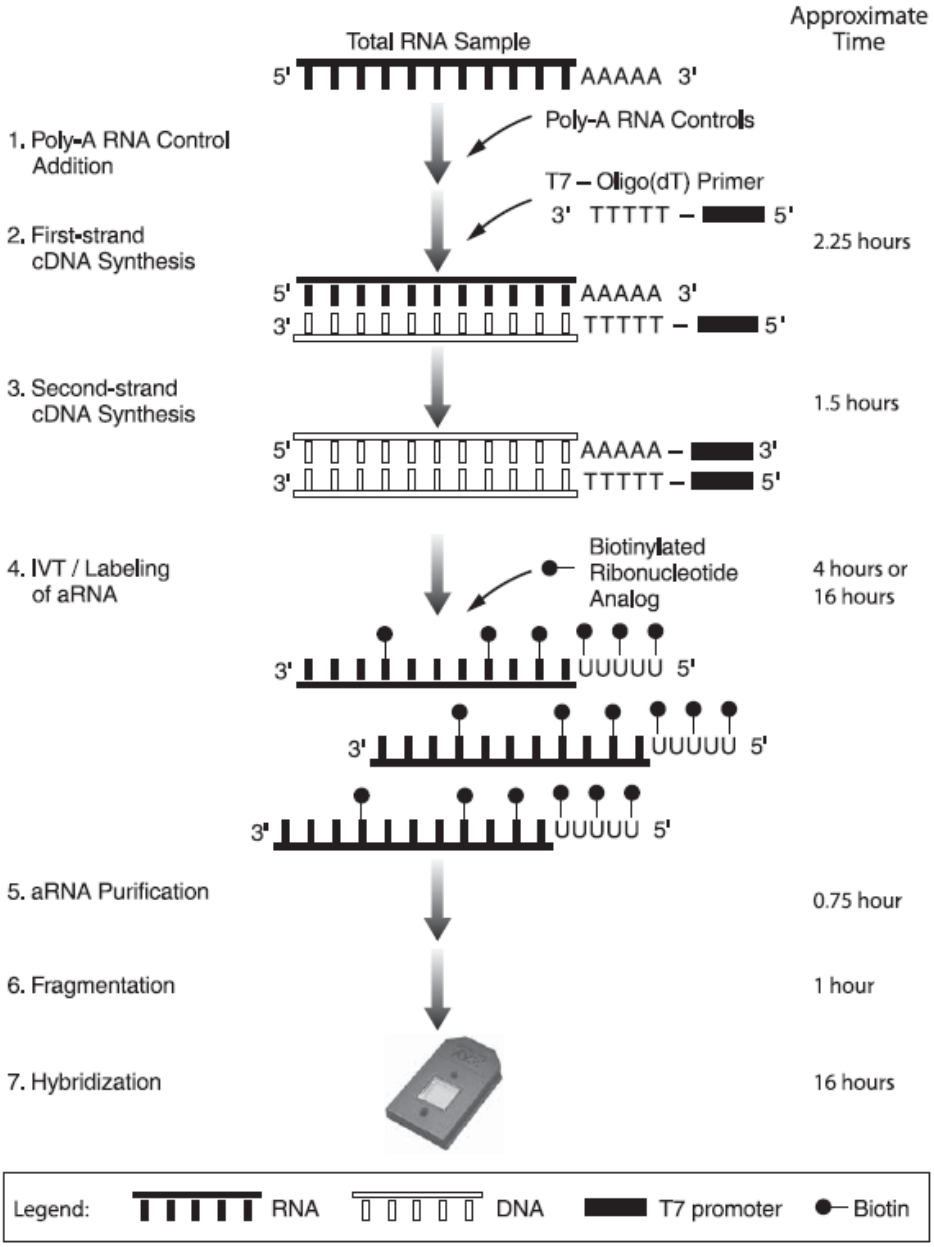

Fig.3: Demonstrating the 7 singe steps of the Affymetrix GeneChip 3'IVT Express KIT assay
1. Poly-A RNA control addition. 2. Reverse Transcription to Synthesize First-Strand cDNA.
3. Second-Strand cDNA Synthesis. 4. In Vitro Transcription to Synthesize Biotin-Modified aRNA with IVT Labeling Master Mix. 5. aRNA Purification. 6. Fragmentation. 7. Hybridization
(http://www.affymetrix.com/_media/images/figures/ht_expresskit_labeling_assay_lg.jpg 21.03.2013).

2.4.1 Poly-A RNA control:

The poly-A RNA controls provide exogenous positive controls to check the eukaryotic target labeling process.

For preparing the poly-A RNA dilution the Poly-A Control Stock and the Poly-A control Dilution (Dil) Buffer (GeneChip IVT Express Kit) were required. 2 µl of the poly-A control stock were put in a 2ml tube and then mixed with 38µl of the poly-A control Dil buffer for the first dilution (1:20) by repetitive pipetting and vortexing. Then the 2 µl of the first dilution were removed and added with 98 µl of Poly-Control Dil Buffer in a fresh set of tubes to mix the second dilution (1:50) by repetitive pipetting and vortexing. The next step was again to add 2 µl of the second dilution to 98 µl of Poly-A Control Dil Buffer in a fresh set of tubes to create the third dilution (1:50). To mix the fourth and final dilution 2 µl of the third dilution was mixed with the 18µl of Poly-A Control Dil Buffer by pipetting and vortexing (1:10). Of the fourth dilution 2µl were added to 100 ng of total RNA for each sample. At the end 2-3 µl of nuclease free water was added to achieve a working volume of 5 µl.

2.4.2 Reverse Transcription to synthesize First and Second-Strand cDNA:

After thawing the First-Strand Buffer Mix and First-Strand Enzyme Mix they were put on ice and mixed together 4µl and 1 µl respectively to assemble the First-Strand Master Mix in a nuclease-free tube by vortexing and briefly centrifugation for 5 seconds.

Meanwhile a 96 well plate was put on ice, 5 µl First-Strand Master Mix was placed in individual wells, one well presenting one patient sample, followed by addition of 5 µl of the RNA/poly-A control mixture to a total volume of 10 µl. After gently vortexing the plate was put for 2 hours at 42°C in a thermal cycler for incubation. After vortexing and briefly centrifugation the plate was put on ice followed by Second-Strand c-DNA synthesis. Like mentioned above, we prepared the Second-Strand Master Mix in a nuclease-free tube with 13 µl nuclease free water, 5µl Second-Strand Buffer Mix and 2 µl Second-Strand Enzyme Mix (from Kit box 2) to a total of 20 µl volume for each sample. After mixing and centrifugation gently the collected 20 µl Second-Strand Master Mix from the bottom of the tube was transferred to each cDNA sample followed by vortexing and briefly centrifugation. The samples were placed in a 16°C thermal cycler block for 1 hour incubation time and followed by a 65°C incubation for 10 min. Subsequently, the samples were centrifuged briefly and stored at -20°C overnight.

2.4.3 In Vitro Transcription to Synthesize Labeled aRNA:

The double-stranded cDNA was taken out of the freezer for thawing and immediately put on ice. For the preparation of the IVT Master Mix (Kit box 2 reagents) a nuclease-free tube was used and the following reagents. IVT Biotin Label 4 µl, IVT Labeling Buffer 20µl and IVT Enzyme Mix 6 µl were added and mixed (vortexing and centrifugation) for a single reaction. The 30 µl of the IVT Master Mix were transferred to each double-stranded cDNA sample and mixed together by gently vortexing and centrifugation. Once composed the plate was put into the thermal cycler for 16 hours incubation at 40°C. The assemble aRNA was put on ice immediately.

2.4.4 aRNA Purification:

Following the aRNA synthesis purification is required through removing enzymes, salt and unincorporated nucleotides. At first the aRNA Binding Mix was assembled at room temperature within a nuclease-free tube by adding RNA Binding Beads and aRNA Binding Buffer Concentrate 10 µl and 50 µl for each sample respectively. The aRNA Binding Mix (60 µl) was then added to each sample followed by transferring the mixture into a well of U-Bottom Plate and mixing it by pipetting up and down several times. 120 µl of 100% ethanol were then transferred to each sample and mixed together through pipetting up and down for several times. In the next step the U-Bottom Plate was put on a Lab-Line Titer Plate Shaker (mode->gently) for about 2-3 min, though the aRNA is able to bind to the RNA Binding Beads. The plate was then attached to a magnetic stand for capturing the magnetic beads for about 5-6 min until all beads had been captured. The supernatant were carefully removed without messing up the magnetic beads and the plate was unattached from the magnetic stand. For bead washing 100 µl of the aRNA Wash Solution (prior mixed with 8ml ethanol) was transferred to each sample and shaken (mode-> moderate) for 1 min on the Lab-Line Titer Plate Shaker. The plate was moved again on the magnetic stand to capture the RNA Binding Beads and removing the supernatant as mentioned above. This step was performed twice and at the end the plate was put on the Lab-Line Titer Plate shaker (mode-> vigorous) for 1 min to evaporate residual ethanol from the beads.

The purified aRNA was eluted from the RNA Binding Beads by transferring 50 µl aRNA Elution Solution, preheated to 50-60°C for about 10-15 min, to each sample. The plate was then shaken (mode-> vigorously) for about 3-4 min until the RNA Binding Beads were fully

distributed. For RNA Binding Beads capturing the plate was attached to the magnetic stand again and the aRNA containing supernatant were removed and put into a nuclease-free PCR tube for each sample.

2.4.5 Evaluation and Fragmentation of aRNA:

After the purification the concentration of the aRNA solution was determined by checking the absorbance at 260 nm for 2 µl aRNA directly by using a NanoDrop spectrophotometer. For providing an optimal assay sensitivity fragmentation of the aRNA before the hybridization onto GeneChip probe array is required. For the fragmentation reaction 15 µl of aRNA, 8 µl Array Fragmentation Buffer and around 18 µl nuclease-free water for a final volume of 40 µl are mixed together (vortexing and pipetting up and down for several times). The reaction was incubated for 35 minutes at 94°C and placed on ice immediately. The fragmentation reaction products were analyzed by running a 300 ng sample of the reaction on the Agilent bioanalyzer using Agilent RNA 6000 Nano Kit. The reaction produces a distribution of 35-200 nt aRNA fragments with a peak at approximately 100-120 nt. The fragmented undiluted aRNA samples were stored at -20 °C until the hybridization to GeneChip Human Genome U133 Plus 2.0 expression arrays were executed.

2.4.6 Hybridization:

Microarray hybridization required the GeneChip Hybridization, Wash and Stain Kit. The 49(standard) array format was executed by mixing the following reagents together for each patient sample: 12.5 µg (=33.3 µl) aRNA, 4.2 µl Control Oligonucleotide B2, 125 µl 20x Hybridization Controls (preheated to 65°C for 5 min), 125 µl 2x Hybridization Mix, 25 µl DMSO (DMSO was thawed prior to use and then stored at room temperature), 50 µl Nuclease-free Water for a total volume of 250 µl. The probe arrays were equilibrated to room temperature before use due to the potential risk of cracking the rubber septa which can lead to leaks. The mixed hybridization cocktail was heated to 99°C for 5 min in a heat block. During that time the arrays were wetted with 200 µl of the Pre-Hybridization Mix by injecting it through the loading septa (see graphic below).

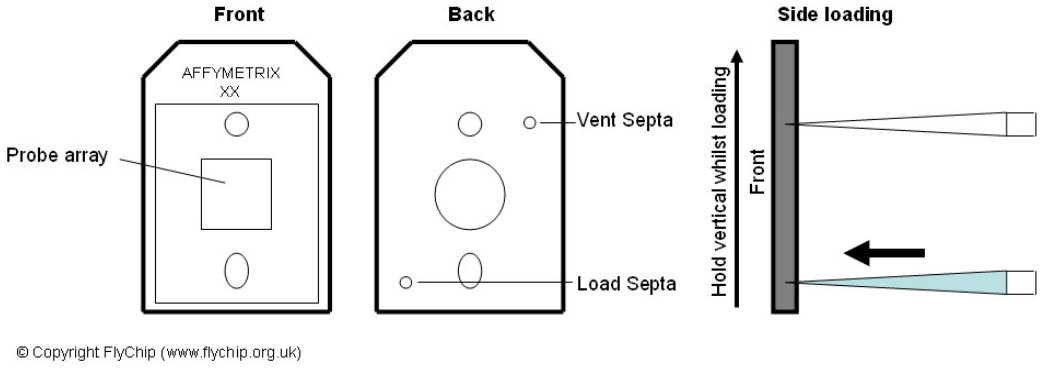

Fig.4: Arrays are wetted with 200 µl of the Pre-Hybridization Mix by injecting it through the loading septa (http://www.flychip.org.uk/protocols/chip/affy_chip.php 18.10.2013).

The filled arrays were incubated at 45°C for 10 min in an oven with rotation. During this time the heated hybridization cocktail was put on heat block for 5 min at 45 °C. After incubation the hybridization cocktail was spun in a microcentrifuge for 5 min for collection insoluble material. The arrays were removed from the oven and ventilated with a clean pipette tip and the Pre-Hybridization Mix was aspirated with a micropipettor and refilled with 200 µl of the cleaned hybridization cocktail (50µl overlap were stored at -20°C). The rubber septa were sealed with a tape. The samples were then transferred to the hybridization oven set to 45°C with a rotation of 60 per min for 16 hours.

2.4.7 GeneChip Expression Washing, Staining and Scanning:

Before washing, staining and scanning each probe array a sample file had to be registered in Affymetrix GeneChip Command Console (AGCC) followed the AGCC user manual section “Creating and Editing Sample Files”. The Fluidics Station 450/250 was used for washing and staining the probe arrays. The Fluidics Station was prepared by filling the intake buffer reservoirs with Wash A and B solutions, filling the water reservoir with deionized water, and placing 3 empty 1.5 ml microfuge tubes into the stain holders followed by running the Prime_450 maintenance protocol from the AGCC Fluidics Control. Meanwhile after 16 hours of hybridization the arrays were taken out of the oven and vented by inserting a pipette tip into one of the septa, while extracting the hybridization cocktail through the other septa.

The array was refilled completely with the appropriate volume (250 µl) of Wash Buffer A. Prior to the washing and staining the stain reagents were prepared like listed below:

- 600µl of Stain Cocktail 1 (light sensitive) into a 1.5ml amber microcentrifuge vial
- 600µl of Stain Cocktail 2 into a 1.5ml clear microcentrifuge vial
- 800µl of Array Holding Buffer into a 1.5 ml clear microcentrifuge vial

For the staining and washing process the AGCC Fluidics Control Software were opened and the required approaches were adjusted so the program could be started. The arrays were put into the designated module. The current microcentrifuge vials were removed from the sample holder and were replaced by the earlier prepared stain reagents like Stain Cocktail 1 in sample holder 1, Stain Cocktail 2 in sample holder 2 and the Array Holding Buffer in sample holder 3. The needle lever was pressed down and the run was started. After about 90 min the program was complete and the probe arrays were taken out and checked for air bubbles followed by scanning by using the Affymetrix GeneChip Scanner and the GenePix Pro 6.0 software. After the scan the probe array images (.dat file) were ready for analysis.

2.5 qRT-PCR:

Five different genes were randomly chosen for qRT-PCR analysis for result evaluation of the GEP namely: *MSI2, ITGB2, MUM1, RBPJ and TRPS1*.

For the qRT-PCR establishment primers for the mentioned genes were designed by using Primer3 input (http://frodo.wi.mit.edu/) and reviewed with UCSC genome Bioinformatics (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start). Before applying the patient samples the primers were tested with a regular PCR using cDNA of the cell lines KG-1 and HL-60. These cell lines were chosen based on the expression of the respective genes in the gene expression database (http://www.ebi.ac.uk/gxa/). For the primer evaluation an agarose gel electrophoresis was performed. For this 2 mg of agarose was put into 100ml of buffer and heated in a microwave for dissolving the agarose. After cooling down to 60°C five drops of ethidium bromide were added and then poured into a gel electrophoresis form.

For the PCR reaction the following reagents for a single reaction were mixed together: 5 µl 10x PCR buffer, 1 µl dNTP Mix, 1 µl primer forward and reverse, 40 µl water followed by vortexing

the mix. Then, 0.25 µl of HotStar Taq Polymerase was added to each reaction. For the next step 2 µl of the cell line cDNA was put into the mix and put on the thermal cycler for 40 cycles, each cycle consisting of 30 sec denaturation at 95°C, 30 sec primer-annealing at 60°C and 60 sec primer-extension at 72°C. After this was finished 10µl of 6x loading buffer was added to each reaction and at end pipetted on the gel. The agarose gel containing each PCR reaction in a single pocket was then put into a buffer filled box and the electric field was applied via the power supply machine, put on 125 mV. After 45 min the gel was taken out under the UV light for examination the gene bands.

After the successful primer validation qRT-PCR analysis of 14 patient samples (6 responders and 8 none responders), using KG-1 and HL-60 cell lines as standard curve, was performed. The patient RNA samples were transcribed into cDNA by using the Super Script III protocol. For each reaction 1 µg of patient RNA was mixed with 1 µl of random hexamer primers and dNTP mix. Diethylpyrocarbonat (DEPC) treated water was added to adjust to a total volume of 10 µl. The RNA-Primer mix was then incubated for 5 min at 65°C. During that time the DNA-synthesis mix was made by mixing 2 µl 10x buffer, 4 µl 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl RNASE Out (40 U/µl) and 1 µl Superscript III RT (200U/µl) for a volume of 10 µl per reaction. The mixes were added together and incubated for 10 min at 25°C, followed by 50 min at 65°C and at the end incubated for 5 min at 85°C for transcription. Taken out of the heat block 2 µl RNASE-H for RNA degradation was added and the mix was heated for 20mins at 37°C and later put on ice. For the RT-PCR gene expression validation a TaqMan7900HT Biosystem and a FAST SYBR Green Master Mix were applied.

The standard curve was established by running the 5 genes plus a housekeeping gene control with ß-actin on a 96 well plate. Each row presented a gene with its respective cell line (figure 5). Into one well 15µl of mix was pipetted including 10 µl 2x SYBR Green Mix, 1 µl primer left and right and 3 µl of water plus 5 µl of template (cell line cDNA or patient samples). After all wells had been filled up an optical adhesive film was used for covering the surface. Due to the long PCR products with a maximum of 295 bp (for *RBPJ*), the annealing time during the melting curve was increased. For details see table below.

Tab.8: Thermal cycler conditions for each cycle for RT-PCR gene expression analysis

Step	Temperature (°C)	Time in seconds	
AmpliTaq Fast DNA Polymerase	95	20	hold
denature	95	5	40 cycles
anneal/extend	60	20	Hold (dissociation step)
measure	depending on primer	20	
dissociation curve	95	15	
	65	20	
	90	20	

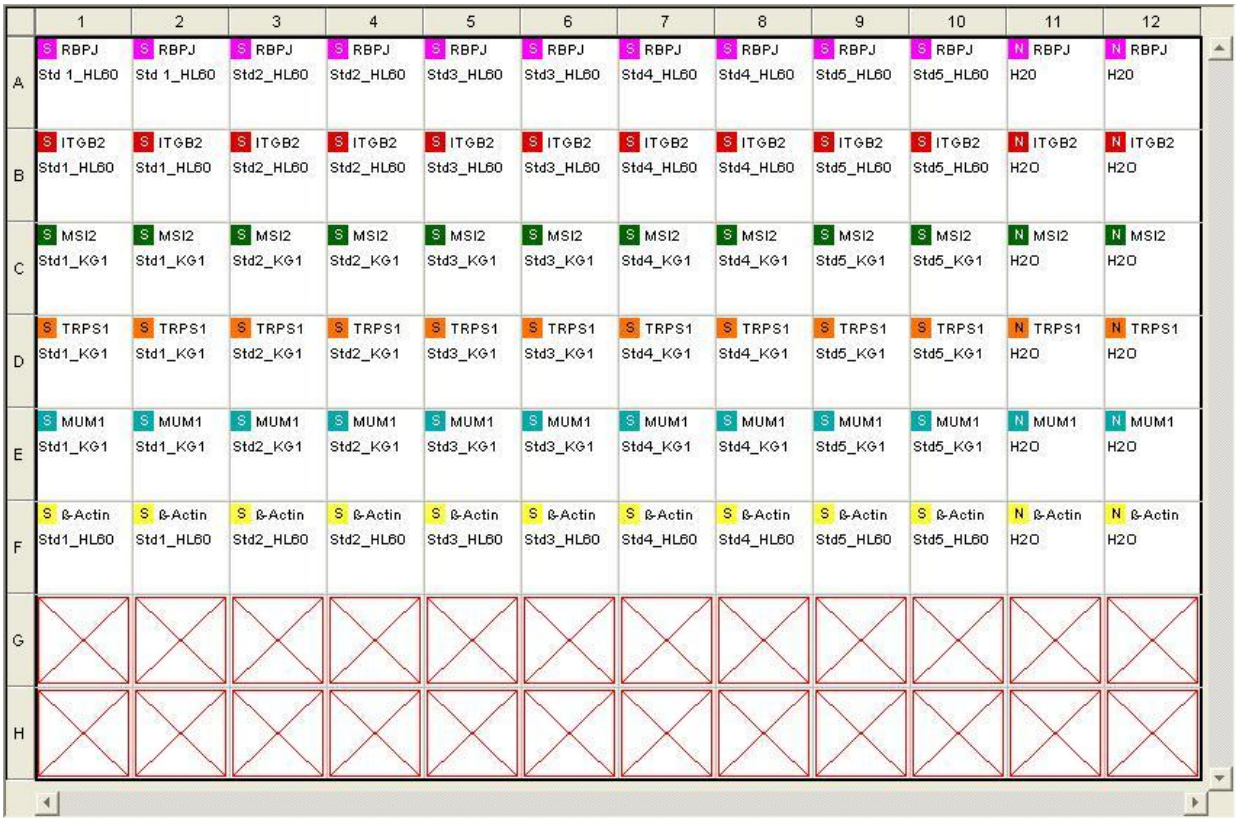

Fig.5: Each row represents a gene as listed above. In the first two wells of each row undiluted cell line cDNA (5 µl) was added to the SYBR Green Mix, each following two wells were diluted 1:10 based on the concentration of the preceding wells. The last two wells 11/12 were mixed with water and were therefore negative controls. The qRT-PCR run was programmed with the 7900HT sequence detection system software V 2.3. (ITGB2= integrine beta 2; MUM1= multiple myeloma oncogene 1; MSI2= musashi 2; RBPJ= recombination signal binding protein; TRPS1= trichorhinophalangeal syndrome I)

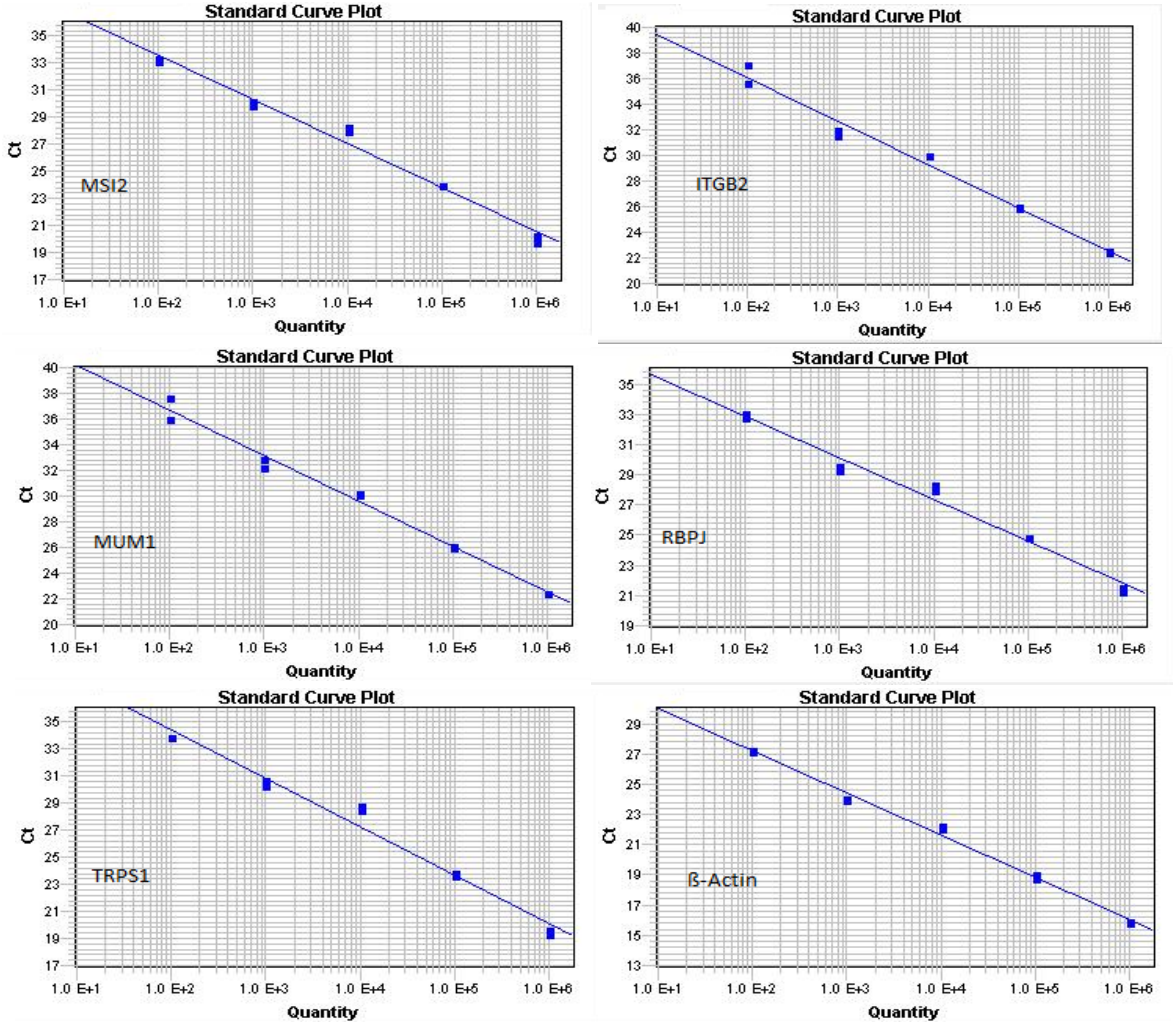

Fig.6: Standard curve execution for each gene applying the sequence detection system software V 2.3 (ITGB2= integrine beta 2; MUM1= multiple myeloma oncogene 1; MSI2= musashi 2; RBPJ= recombination signal binding protein; TRPS1= trichorhinophalangeal syndrome I)

Following the successful execution of the standard curve for each gene, a total of 5 96 wells plate 1 for each gene with ß-actin as a control on each plate were pipetted. In the first row the respective gene with its cell line cDNA and serial dilution were put in, followed by patient samples in duplicates (for example see figure below of *RBPJ*). The mixture in each well was prepared as describe above: with 15 µl of mix including 10 µl 2x SYBR GreenMix, 1 µl primer left and right and 3 µl of water plus 5µl of template (cell line cDNA or patient samples). After the pipetting the 96 well plate was put in the 7900HT Taqman for qRT-PCR analysis.

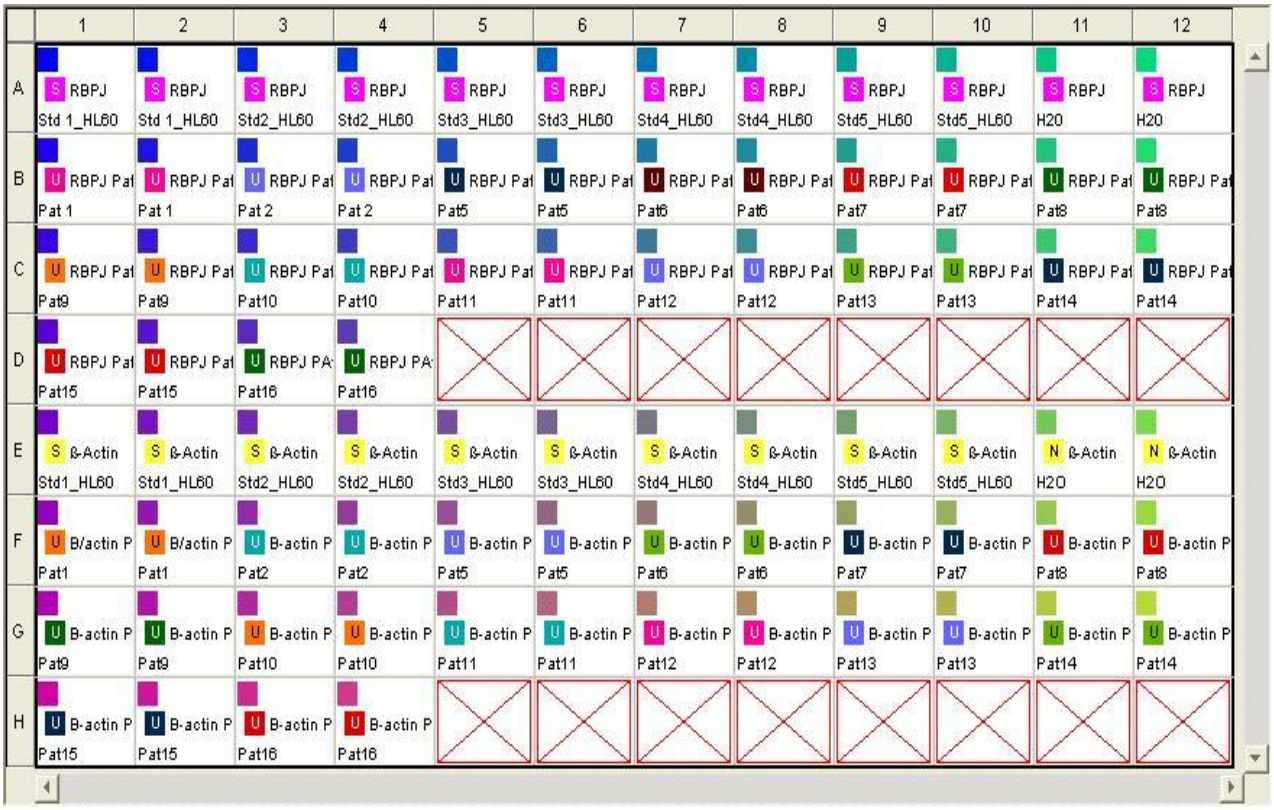

Fig.7: Example of the 96 well plate executing qRT-PCR of the patient samples with the first row representing the gene with the according cell line followed by the patient samples in duplicates. (ITGB2= integrine beta 2; MUM1= multiple myeloma oncogene 1; MSI2= musashi 2; RBPJ= recombination signal binding protein; TRPS1= trichorhinophalangeal syndrome I)

2.6 Data analysis of microarrays and qRT-PCR

Microarray data analysis:

For the microarray data analyses the BRB array tool software version 4.3.0 beta3 developed by Dr. Richard Simon and BRB-ArrayTools Development Team was used (available at -> http://linus.nci.nih.gov/BRB-ArrayTools.html). The cell files were filtered by using the JustRMA algorithm and filtering criteria as previously reported [22].

Differentially expressed genes between two patient groups were discovered through class comparison analyses. The type of univariate test used was the two-sample t-test separately for each gene using the normalized log-ratios for RNA microarray. A gene was defined as statistical significant in one of these groups if a p-value of <0.05 was achieved. Results of class

comparison analysis were visualized by supervised hierarchical cluster analysis of the most significant differentially expressed genes using Cluster, and the results were visualized with Tree-View (http://www.eisenlab.org/eisen/?page_id=42, 01.08.2013) [64].

Besides gene class comparison analysis, we investigated pathways which contain more differentially expressed genes than expected by chance using the BRB Pathway Comparison tool. This tool identifies genes represented in the corresponding pathway and the statistical significance p-value defined as <0.05 for each gene in the group. The least squares/Kolmogorov-Smirnov (LS/KS) permutation and the Efron-Tibshirani's GSA max-mean test were applied summarizing the p-values for genes for the respective pathway. For identification of genes correlating with the survival a cox proportional hazards model was performed ($p<0.05$). With a gene set enrichment analysis for biological mechanisms using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov; 01.08.2013) an enrichment of genes belonging to functional gene ontology (GO) groups could be identified. A GO category was accepted as statistical significant if $p<0.05$ using Fisher's exact test and if 5 or more genes were involved. For comparison and correlation of microarray with the qRT-PCR results, the mean of all raw data was calculated and then subtracted of the value of each patient (log_2 transformed microarray data were mean-centered, see below).

qRT-PCR result analysis:

For the analysis of qRT-PCR results the raw data had to undergo multiple calculations in order to allow the evaluation of the differential expression of the respective genes, especially in comparison with the microarray results.

The cycle threshold (CT) is a theoretical value that describes the exponential growth of the qRT-PCR curves, reflecting the quantification of the DNA molecules for which the fluorescence is exponential for the first time over the background value. The CT is already given by the SDS software V 2.3 (company) if the qRT-PCR run analysis is performed. To evaluate the specific expression of the gene it has to be put in relation to a house keeping gene (HKG) that serves as reference gene (-> investigated gene value/HKG value). The HKG is gene is constitutively expressed in all cell types of an organism under physiological and pathophysiological

conditions. In our study the HKG ß-Actin was chosen. Taken the raw quantity (Qty) expression value of the duplicates, the mean was calculated and taken into the following mathematic steps to evaluate the expression for each patient sample:

$$template\ quantity = \frac{Qty\ mean\ of\ gene}{Qty\ mean\ of\ reference\ gene}$$

followed by the logarithm of the basis 2

$$\log_2(template\ quantity)$$

The log2(template quantity) value of each patient was added together and dived by the number of patients leading to the mean of log2(template quantity). The mean was then subtracted of each individual log2(template quantity) value leading the expression result that is valid for taken in comparison to the equivalent GEP result. For comparison and setting these results in correlation the Pearson-Test was used.

2.7 Chemicals, Reagents and Instruments:

The below listed chemicals and instruments were obtained from the respective suppliers and used for the laboratory experiments:

For gene expression analysis:

Tab.9: Affymetrox GeneChip 3'IVT Express Kit Components

Affymetrix GeneChip® 3' IVT Express Kit Components	
aRNA Binding Buffer Concentrate	Affymetrix ®
RNA Binding Beads	Affymetrix ®
aRNA Wash Solution Concentrate	Affymetrix ®
aRNA Elution Solution	Affymetrix ®
Nuclease-free Water	Affymetrix ®
5X Array Fragmentation Buffer	Affymetrix ®
8-Strip PCR Tubes & Caps (0.2 mL)	Affymetrix ®
U-Bottom Plate	Affymetrix ®
Reservoir	Affymetrix ®
First-Strand Enzyme Mix	Affymetrix ®
First-Strand Buffer Mix	Affymetrix ®
Second-Strand Enzyme Mix	Affymetrix ®
Second-Strand Buffer Mix	Affymetrix ®
IVT Enzyme Mix	Affymetrix ®
IVT Labeling Buffer	Affymetrix ®
IVT Biotin Label	Affymetrix ®
Control RNA (1 mg/mL HeLa total RNA)	Affymetrix ®
Nuclease-free Water	Affymetrix ®
Poly-A Control Stock	Affymetrix ®
Poly-A Control Dilution Buffer	Affymetrix ®
20X Hybridization Controls	Affymetrix ®
Control Oligo B2	Affymetrix ®

Tab.10: Affymetrix GeneChip Hybdridization Wash and Stain Kit Components

Affymetrix GeneChip® Hybridization Wash and Stain Kit	
Pre-Hybridization Mix	Affymetrix ®
2X Hybridization Mix	Affymetrix ®
DMSO	Affymetrix ®
Nuclease-free water	Affymetrix ®
Stain Cocktail 1	Affymetrix ®
Stain Cocktail 2	Affymetrix ®
Array Holding Buffer	Affymetrix ®
Wash Buffer A	Affymetrix ®
Wash Buffer B	Affymetrix ®

Tab.11: Applied Instruments

Instruments	Supplier
GeneChip® Hybridization Oven 640	Affymetrix®
GeneChip® Fluidics Station 450	Affymetrix®
GeneChip® Scanner 3000	Affymetrix®

For qRT-PCR analysis:

Tab.12: Primer (produced by Eurofins MWG Synthesis GmbH, Germany) (ITGB2= integrine beta 2; MUM1= multiple myeloma oncogene 1; MSI2= musashi 2; RBPJ= recombination signal binding protein; TRPS1= trichorhinophalangeal syndrome I):

Gene	Primersequenz 5`->3`		Product length	Cell line
	forward	reverse		
MSI2	GAAAGCTCAGCCGAAAGAA	GTTGGAGCCTGATCCTCTTG	251bp	KG-1
ITGB2	GATGGACCGCTACCTCATCT	GCGCTCTTGAAAAGGGGATT	228bp	KG-1
MUM1	GGTTCATTCTGGACGTGCTT	TGTACTTCTCCTCAGCCGTC	250bp	KG-1
RBPJ	CTTGCCCCAGTCACTCCTG	GGGACGACACAGAGCATACT	176bp	HL-60
TRPS1	TCCAAGGATGAATCCCAGTC	TGACTTGCTCCTCATTGCTG	295bp	HL-60

Tab.13: Chemicals/Instruments:

Reagent	Supplier
10x PCR Puffer	Qiagen N.V., Netherlands
Hot Star Taq DNA Polymerase	Qiagen N.V., Netherlands
dNTP mix	Qiagen N.V., Netherlands
Nuclease free Water	Ambion Inc; USA
Agarose 1%	BioWhittaker Molecular Applications, USA
Ethiumbromid	Carl Roth GmbH, Germany
Random hexamers (50ng/µL)	Invitrogen by life technologies; USA
10x RT buffer	Invitrogen by life technologies; USA
25 mM MgCl2	Invitrogen by life technologies; USA
0.1 M DTT	Invitrogen by life technologies; USA
10 mM dNTP mix	Invitrogen by life technologies; USA
SuperScript III RT (200 U/µL)	Invitrogen by life technologies; USA
RNase OUT (40U/µL)	Invitrogen by life technologies; USA
DEPC treated water	Invitrogen by life technologies; USA
Rnase H (2U/µL)	Invitrogen by life technologies; USA
Fast SYBR Green Master Mix	Applied Biosystems, USA
MicroAmp Fast Optical 96-Well Reaction Plate	Applied Biosystems, USA
MicroAmp 96-Well Optical Adhesive Film	Applied Biosystems, USA

3 Results

3.1 Analysis of patient outcome data:

3.1.1 Response to DAC:

Often elderly AML patients are not eligible for intensive induction therapy therefore DAC may be a good option for treatment (see above). But so far there are no valuable biomarkers available that may predict response to DAC sufficiently. Due to this purpose a gene expression analysis of 46 patients treated with DAC was executed and these results were set in association to the clinical data.

However, only for 36 patients survival data and response rate were accessible: 4 died within the first course of application and therefore were not accessible for response analysis and 6 patients were lost to follow-up. The objective response (PR and CR) was seen in 25% (n=9) of cases: complete remission n=4 (11%), partial remission n=5 (14%). An additional n=8 (22%) patients achieved an ALE as best response resulting in a total response rate of 47%. Eleven patients (31%) obtained stable disease and 8 had progressive disease (22%). The 6 week death rate for all 46 patients, defined as early death by the study group, was 9% (n=4). Of the 17 patients showing a response (CR, PR or ALE), 89% achieved a response after the first 2 cycles.

Tab.14: Response rate of DAC treated patients (ALE= anti leukemic effect, CR= complete remission; PD= progressive disease; PR= partial remission; SD= stable disease)

Response	Rate n. (%)
Response	17 (47)
CR	4 (11)
PR	5 (14)
ALE	8 (22)
No response	19 (53)
SD	11 (31)
PD	8 (22)

The analysis of clinical data (LDH, WBC, platelet count, hemoglobin, blast count) at baseline did not show any statistical difference between the response and no response patient cohorts (data shown in table 15). In univariate analysis no correlation between clinical data at baseline including prior MDS, cytogenetic risk, ECOG status, genders and FAB classification and response to DAC was detected. However, there was a trend of the cytogenetic high risk group being associated with DAC response (p-value = 0.096), although due to the limited number of cases this association remains elusive.

Tab.15: Comparison of preclinical data between responder and non responder. (DNMT3A= DNA methyltransferase, FAB= French-American-British;MDS=myeloid dysplastic syndrome NPM1= nucleophosmin)

Parameters mean(range)	Non responder	Responder	p-value
Age (years)	72 (62-83)	72 (61-85)	0.75
>75	4	5	0.71
<75	15	12	
Lactat dehydrogenase (U/L)	390 (125-1720)	277 (131-678)	0.20
<300	9	13	0.09
>300	10	4	
White blood cell count (G/l)	18.9 (0.7-186.4)	6.2 (0.8-20.6)	0.21
<5	10	6	0.33
>5	9	11	
Platelet (G/l)	67 (13-179)	91 (13-474)	0.41
<50	9	7	0.75
>50	10	10	
Hemoglobin (g/dl)	9.4 (7.9-11.7)	8.6 (5.7-11.1)	0.13
<10	14	13	
>10	5	4	
Peripheral blast count %	19.8 (0-71)	14.4 (0-64)	0.44
Bone marrow blast count %	63.8 (32-96)	56 (0-95)	0.47
<50	7	7	
>50	9	9	
Missing	2		

Prior MDS - n. (%)			0.56
Yes	6 (32)	7 (41)	
No	13 (68)	10 (59)	
Cytogenetic risk – n. (%)			0.096
High	3 (16)	7 (41)	
Intermediate	12 (63)	7 (41)	
Missing	4	3	
Performance status – n. (%)			0.14
0	1 (5)	3 (18)	
1	12 (63)	12 (71)	
2	5 (27)	2 (11)	
Missing	1 (5)	/	
Gender – n. (%)			0.56
Male	13 (68)	10 (59)	
Female	6 (32)	7 (41)	
FAB Classification – n. (%)			0.94
M0	/	2 (12)	
M1	8 (42)	6 (35)	
M2	7 (36)	5 (29)	
M3	/	/	
M4	2 (11)	1 (6)	
M5	/	/	
M6	/	/	
M7	/	1 (6)	
Missing	2 (11)	2 (12)	
DNMT3A mutation (%)			0.66
No	15 (79)	14 (88)	
Yes	4 (11)	2 (12)	
Missing	/	1	
NPM1 mutation (%)			1.0
No	16 (84)	14 (88)	
Yes	3 (16)	2 (12)	
Missing	/	1	

3.1.2 Survival analysis:

In our small cohort the median overall survival (OS) time of the patient cohort was 150 days with a 1 year survival rate of 25% and a 2 year survival rate of just 5%. Patients who achieved a response had a statistical significant (p=0.045) (figure 8) increased survival time with a median OS of 338 and 137 days for responders and non-responders and a mean OS of 388 and 243 days, respectively. Correlation between pretreatment clinical data and OS was seen for age and bone marrow blast percentage. The entire data are shown in the following tables below.

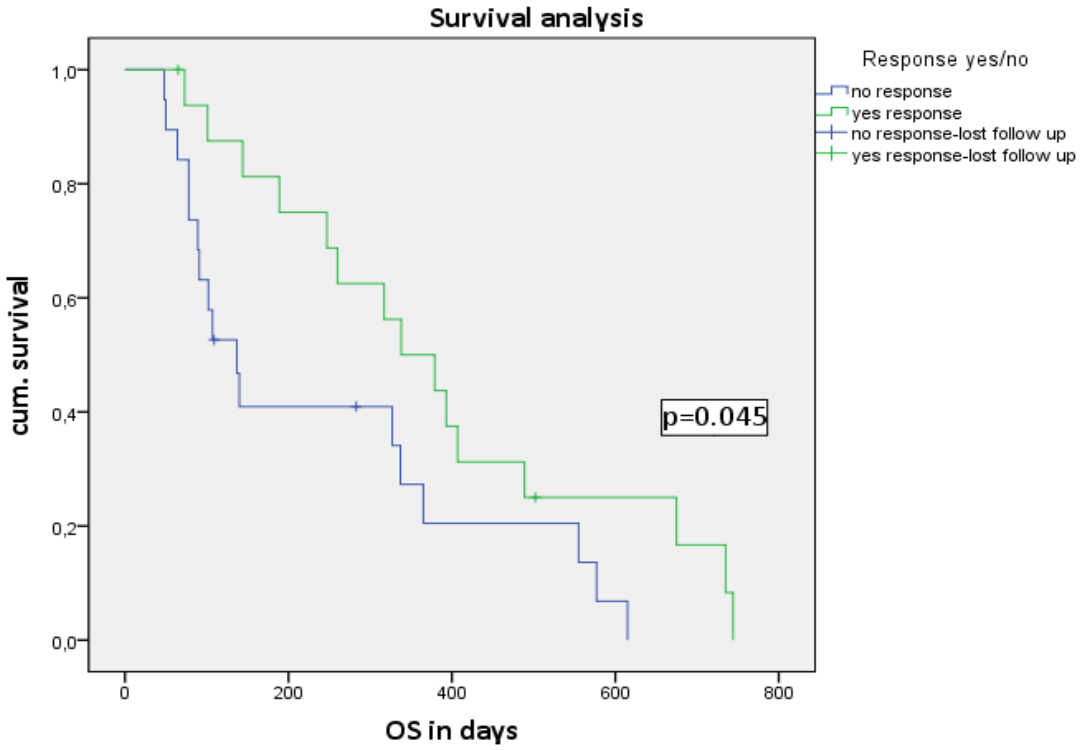

Fig.8: Kaplan Meier curve displaying the overall survival (OS) of the 2 groups (responders and non-responders). Responder (green) had a significant better OS than the non-responder (blue ; p-value = 0.045).

Tab.16: Patients overall survival according to response (OS=overall survival)

Response group	OS in days median	OS in days mean (range)	1 year survival rate
all patients	150	263 (3-744)	27%
response	338	388 (65-744) **(p=0.045)**	47%**(p=0.16)**
no response	137	243 (48-615)	21%
ALE	317	249 (73-393)	
PR	407	453 (189-735)	
CR	556	489 (247-744)	
SD	137	237 (48-615)	
PD	102	176 (78-327	

Tab.17: Patients overall survival according to clinical data.

	OS in days median	OS in days mean	p-value
Age			
>75	103	162 (3-407)	0.052
<75	260	300 (10-744)	
Gender			
male	144	269 (3-744)	0.73
female	189	254 (10–675)	
Performance status			
0+I	247	287 (3-744)	0.21
>II	89	183 (48-615)	
Cytogenetic risk			
High	144	178 (3-502)	0.14
Intermediate	337	300 (10-744)	
White blood cell count (G/l)			
<5,0	144	244 (26–744)	0.98
>5,0	266	276 (3–735)	
Platelet (G/l)			
<50	103	208 (3-735)	0.10
>50	383	323 (10-744)	
Hemoglobin (g/dl)			
<10	140	249 (3-735)	0.31
>10	337	315 (10-744)	
Bone marrow blasts %			
<50	365	373 (73-744)	**0.034**
>50	144	213 (3–675)	
Lactat dehydrogenase (U/L)			
<300	189	274 (3-744)	0.55
>300	137	238 (26-577)	

In comparison to the entire patient cohort treated in this clinical trial only a few differences were seen in our patients, thus we think that our collection of cases is representative not introducing a significant bias in the analysis. For example, the objective response rate was quite similar, 25% in the Ulm cohort of the study and 26% in the entire study cohort. The 6 week death rate was also comparable (9% vs. 12%). In contrast to the trial, we did not observe any significant association between performance status and objective response, as well as cytogenetics and preceding MDS. The median overall survival of the Ulm patients was also similar (150 vs. 165 days), as was the 1 year survival rate (27% vs. 28%). The 2 year survival rate cannot be compared due to the small number of patients in the Ulm cohort.

A decreased survival for patients older than 75 was seen, although not statistically significant, but there was as a strong tendency with a p-value of 0.052 in the Ulm cohort (similar as in the trial). Lubbert et al also mentioned a decreased survival for patients with increased WBC, low platelet counts (< 50 G/l) and LDH serum levels higher than 300 U/L. Most likely due to the small cohort analyzed in our study, this could not be confirmed in our study, but a significant shorter survival was seen in patients with a bone marrow blast count percentage >50% reflecting a higher proliferative potential and a more aggressive or progressed disease. As outlined above statistical analysis for response and survival in association with clinical data are comparable between our selection of cases and the entire study cohort. The differences found are probably just due to the limited number of patients entered in this analysis compared to the entire cohort in the study.

3.2 Results of the gene expression analysis:

First a class comparison analysis between responder and non-responder was executed to determine specific gene expression patterns distinguishing the groups. The genes were then visualized by a supervised hierarchical cluster analysis to distinguish between DAC response and no response cases depending on the expression profile at baseline. In addition, a gene set enrichment analysis for the identification of biological mechanisms of the genes found in the two different groups using DAVID was done as well as executing a pathway comparison analysis to detect distinct pathways that are involved. Finally, a survival analysis was revealed

to identify genes that are associated with survival as well as with treatment response to illustrate the genes that do not only predict response but also may correlate with prolonged remission and overall outcome.

3.2.1 Class comparison analysis:

Due to the lack of valuable biomarkers for DAC response prediction, a class comparison analysis based on the 21054 filtered genes differently expressed between responder and non-responder was executed. A gene was defined as differently expressed if a p-value <0.05 was achieved in a univariate t-test. The aim was to identify certain genes expression profiles for a better understanding of who may benefit from an epigenetic therapeutic approach primarily to DAC in this case. In the analysis a total of 301 genes were significantly associated with either response or no response. For the response group 124 significantly higher expressed genes were identified involving genes like: *ASCL2, INPP5D, SOX4, MSI2, SLC24A3, PRAME, HOXB9, HOXB7, HOXA4, TNSFS9, MSX2* and *MLL.*

Several of these genes were previously described as candidate genes correlating with a decreased remission rate to conventional AML therapy (“7+3”) including *SOX4*, *MSI2*, *MLL* and the homeobox gene family. On the other hand genes representing a functional immune response are included in the response group as well like *MUM1* and *TNSFS9*.

Tab.18: Selection of interesting genes correlating positive with response to DAC= decitabine treatment.

Probe set	Gene Symbol	Gene Name
229638_at	*IRX3*	iroquoishomeobox 3
219090_at	*SLC24A3*	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3
201418_s_at	*SOX4*	SRY (sex determining region Y)-box 4
219541_at	*LIME1*	Lck interacting transmembrane adaptor 1
237180_at	*PSME4*	proteasome (prosome, macropain) activator subunit 4
235439_at	*RBMS2*	RNA binding motif, single stranded interacting protein 2
239232_at	*MSI2*	musashi homolog 2 (Drosophila)
221290_s_at	*MUM1=IRF4*	melanoma associated antigen (mutated) 1
235249_at	*RDH13*	retinol dehydrogenase 13 (all-trans/9-cis)
221690_s_at	*NLRP2*	NLR family, pyrin domain containing 2
210239_at	*IRX5*	iroquoishomeobox 5
36553_at	*ASMTL*	acetylserotonin O-methyltransferase-like
242778_at	*LPXN*	Leupaxin
206170_at	*ADRB2*	adrenergic, beta-2-, receptor, surface
202806_at	*DBN1*	drebrin 1
226461_at	*HOXB9*	homeobox B9
208553_at	*HIST1H1E*	histone cluster 1, H1e
1568943_at	*INPP5D=SHIP1*	inositol polyphosphate-5-phosphatase
204086_at	*PRAME*	preferentially expressed antigen in melanoma
229215_at	*ASCL2*	achaete-scute complex homolog 2
206907_at	*TNFSF9*	tumor necrosis factor (ligand) superfamily, member 9
210319_x_at	*MSX2*	mshhomeobox 2
220546_at	*MLL*	myeloid/lymphoid or mixed-lineage leukemia
204086_at	*PRAME*	preferentially expressed antigen in melanoma
200976_s_at	*TAX1BP1*	Tax1 (human T-cell leukemia virus type I) binding protein 1
206289_at	*HOXA4*	homeobox A4
202332_at	*CSNK1E*	casein kinase 1, epsilon
222113_s_at	*EPS15L1*	epidermal growth factor receptor pathway substrate 15-like 1
244784_at	*DHX57*	DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57
204778_x_at	*HOXB7*	homeobox B7

Next to the genes associated with response to DAC therapy, 177 genes showed a significant $p<0.05$ correlation with no response. Among these genes were: *IFI27, IFI44L, THBS1, FAS, MX1, TRPS1, RBPJ, ITGB2, ADAM9, NOTCH2, PDK4* and *HDAC4.*

As mentioned above genes representing an adequate immune response correlate with response, however genes that rather are associated with an insufficient immune response are found in the no response group involving *IFI27* and *IFI44L*. Genes previously described as a TSG (tumor suppressor gene) genes are quite frequently seen in the group of no response including *FAS, MX1, MXD1* and *FRMD1*.

HDAC4, a histone deacetylase, was also found in this group reflecting the role of epigenetic changes in these cases. Quite interesting is the identification of genes associated directly or indirectly with the NOTCH/WNT signaling pathway including *NOTCH2*, *RBPJ* and *TRPS1*.

Tab.19: Selection of interesting genes correlating positive with no response to DAC=decitabine treatment.

Probe set	Gene Symbol	Gene Name
225897_at	MARCKS	myristoylated alanine-rich protein kinase C substrate
203184_at	FBN2	fibrillin 2
225481_at	FRMD6	FERM domain containing 6
236995_x_at	TFEC	transcription factor EC
202381_at	ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)
204225_at	HDAC4	histone deacetylase 4
210756_s_at	NOTCH2	Notch homolog 2 (Drosophila)
224928_at	SETD7	SET domain containing (lysine methyltransferase) 7
1553685_s_at	SP1	Sp1 transcription factor
225745_at	LRP6	low density lipoprotein receptor-related protein 6
203973_s_at	CEBPD	CCAAT/enhancerbindingprotein (C/EBP), delta
215719_x_at	FAS	Fas (TNF receptor superfamily, member 6)
212195_at	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)
202430 s at	PLSCR1	phospholipid scramblase 1
205407_at	RECK	reversion-inducing-cysteine-rich protein with kazal motifs
207785_s_at	RBPJ	recombination signal binding protein for immunoglobulin kappa J region
202086_at	MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
236988_x_at	ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
225188_at	RAPH1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1
218502_s_at	TRPS1	trichorhinophalangeal syndrome I
226275_at	MXD1	MAX dimerization protein 1
201667_at	GJA1	gap junction protein, alpha 1, 43kDa
204439_at	IFI44L	interferon-induced protein 44-like
225207_at	PDK4	pyruvate dehydrogenase kinase, isozyme 4
202609_at	EPS8	epidermal growth factor receptor pathway substrate 8
200602_at	APP	amyloid beta (A4) precursor protein
227038_at	SGMS2	sphingomyelin synthase 2
201110_s_at	THBS1	thrombospondin 1
1569362_at	ALCAM	activated leukocyte cell adhesion molecule
202411_at	IFI27	interferon, alpha-inducible protein 27

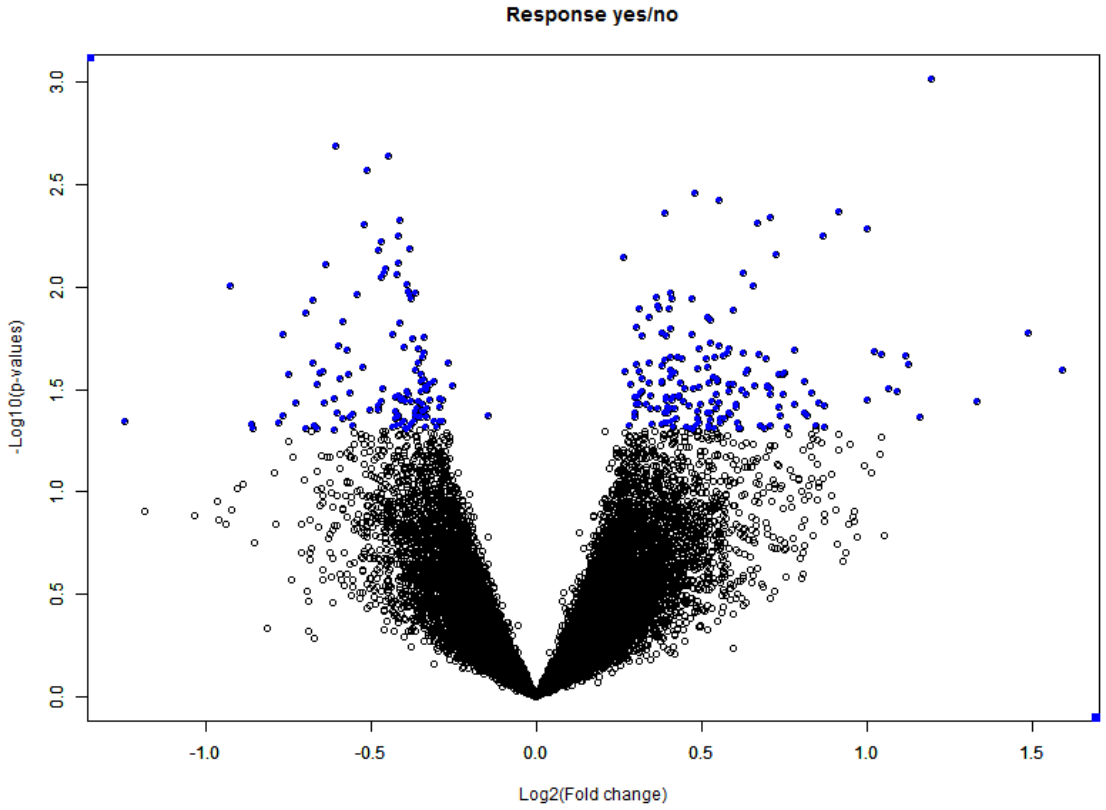

Fig.9 The class comparison analysis identified 301 genes associated with treatment response. In the above volcano dot plot all significant differently expressed genes ($p<0.05$) (right associated with no response; left associated with response) are illustrated as blue dots (each dot is representing a gene). The black dots represent genes associated with either response or no response, which however do not show a significant difference ($p>0.05$).

3.2.2 Supervised cluster analysis:

A supervised cluster analysis of the gene expression profile of the two groups was performed to specify between these two groups by their expression profile to discover a distinct gene patterns.

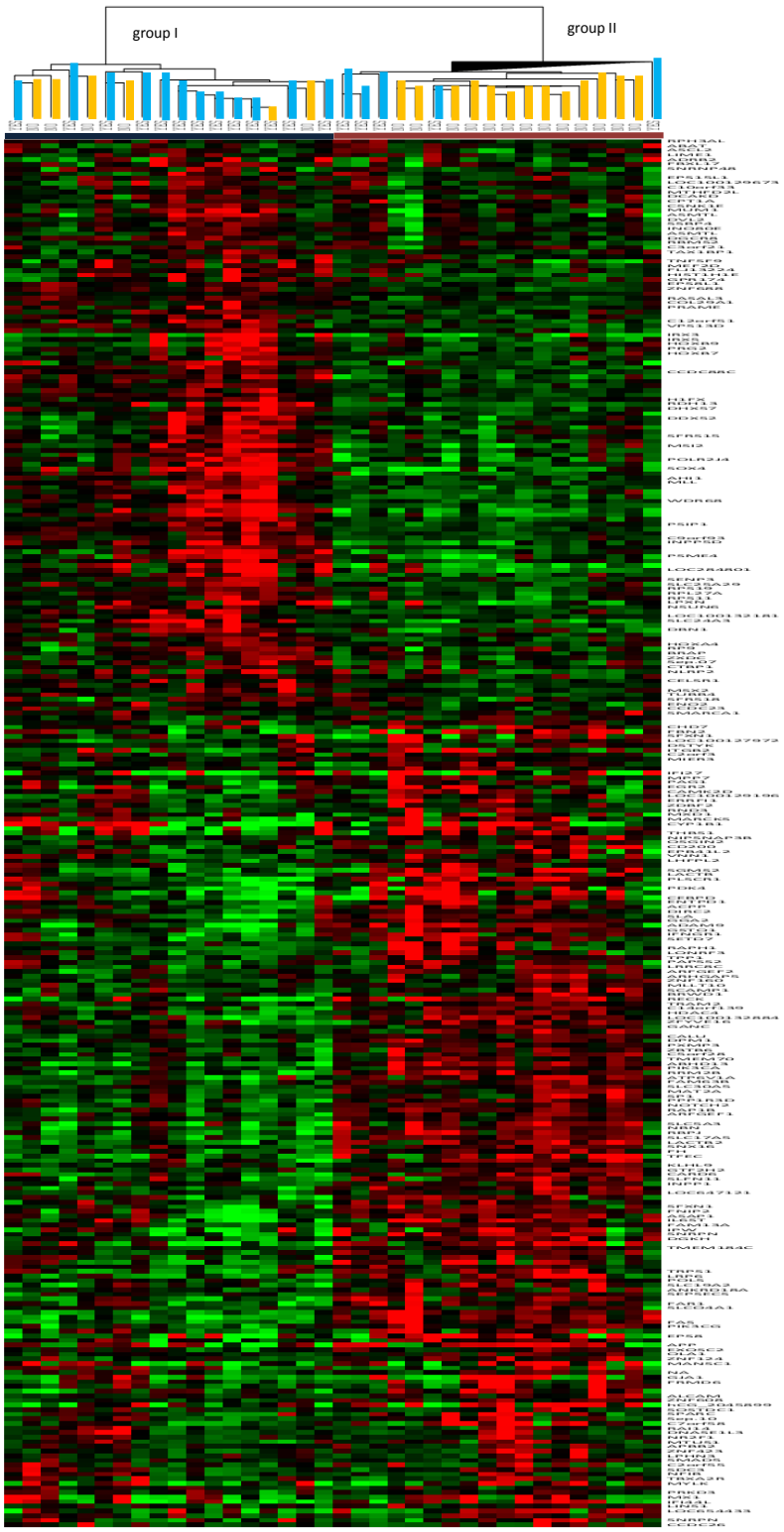

Fig.10: Supervised hierarchical cluster analysis based on the genes correlated with response or resistant disease (as detected by the class comparison analysis); the cluster shows a clear distribution into two distinct groups; group I (dark blue) is characterized by mainly responders (66%), whereas group II (bordeaux red) contains mainly non-responders (72%). The gene expression data are color coded: red reflecting increased expression; green reflecting decreased expression (compared to the mean expression across all leukemias of the respective gene).

3.2.3 Gene expression survival analysis:

To determine genes that are not just associated with response, but that also correlate with increased/decreased survival, a gene expression survival analysis was performed. Survival time was provided in days and the event was defined as death. A total number of 2268 genes were identified, and a comparison of these genes with the 301 genes, showed an overlap of 19 genes were overlapping (shown in table below).

Tab.20: Genes correlating with survival as well as response (HR= hazard ratio).

ProbeSet	HR	p-value	Symbol	Name
219448_at	1.787	0.0002215	TMEM70	transmembrane protein 70
210175_at	2.954	0.0009025	C2orf3	chromosome 2 open reading frame 3
223342_at	2.309	0.00126	RRM2B	ribonucleotidereductase M2 B (TP53 inducible)
242931_at	1.748	0.001862	LONRF3	LON peptidase N-terminal domain and ring finger 3
224928_at	1.945	0.0024004	SETD7	SET domain containing (lysine methyltransferase) 7
207785_s_at	1.841	0.0032775	RBPJ	recombination signal binding protein for immunoglobulin kappa J region
202381_at	1.505	0.0036733	ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)
1569362_at	1.774	0.0053565	ALCAM	activated leukocyte cell adhesion molecule
226275_at	1.773	0.0054814	MXD1	MAX dimerization protein 1
224525_s_at	2.957	0.0057588	OLA1	Obg-like ATPase 1
1552486_s_at	1.82	0.0084898	LACTB	lactamase, beta
209681_at	1.544	0.0112853	SLC19A2	solute carrier family 19 (thiamine transporter), member 2
235348_at	1.434	0.0135488	ABHD13	abhydrolase domain containing 13
204554_at	1.947	0.0142694	PPP1R3D	protein phosphatase 1, regulatory (inhibitor) subunit 3D
202673_at	1.854	0.0173739	DPM1	dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit
219029_at	1.639	0.0227234	C5orf28	chromosome 5 open reading frame 28
236995_x_at	1.377	0.0236905	TFEC	transcription factor EC
227038_at	1.282	0.045907	SGMS2	sphingomyelin synthase 2
213979_s_at	0.499	0.0490352	CTBP1	C-terminal binding protein 1

One of the 19 genes was identified that was accompanied with response and prolonged survival. *CTBP1* is located on Chr4p16 and encodes for a phosphoprotein that functions as

transcriptional repressor and seems to play a role during cellular proliferation. On the other hand 18 of the overlapping genes detected were associated with no response and decreased survival. For example, these genes included *ADAM9*, which is associated with aggressive tumors like in breast cancer. ALCAM, a leukocyte cell adhesion molecule involved in the processes of cell adhesion, migration and was identified as an essential regulator for long-term hematopoietic stem cell engraftment and self-renewal.

RBPJ was the only gene detected in all three analyses, gene class comparison, pathway class comparison and survival analysis. It is essential for the Notch signaling pathway were it functions as a repressor if not bound to Notch proteins and as an activator if bound to Notch proteins. It also recruits chromatin remodeling complexes containing histone deacetylase or histone acetylase proteins.

Additional genes were *OLA1*, a novel DNA damage-regulated nucleocytoplasmic ATPase, which regulates oxidative stress response, is regularly highly expressed in human malignancies. *MXD1* a TSG, antagonizes MYC-mediated transcriptional activation and missense mutations in this gene may influence the pathomechanism of acute leukemia. Other interesting genes included *TFEC* an oncogene, *RRM2B/p53R2* a DNA-hypomethylation independent DAC target gene or *SETD7* a lysine methyltransferase underlining the importance of epigenetic mechanisms have been identified.

3.2.4 Enrichment analysis of distinct gene sets

The enrichment analysis evaluates the quantity of genes of a certain GO group involved in the characteristic signature and compares a potential enrichment to the quantity of all genes of a certain GO group in the entire gene background. By applying a statistical algorithm (Fisher-Exact test) a significant enrichment for genes of a certain GO group/pathway can be detected in a specific gene signature. By using Database for Annotation, Visualization and Integrated Discovery (DAVID) the genes identified through the class comparison characterizing response or no response were screened if an enrichment of genes of certain gene ontology groups could be detected. In addition, a pathway comparison was executed to discover certain signaling pathways involving the previously found genes. The pathway analysis provides a better insight into the pathomechanism affecting the epigenetic therapy.

Tab.21: Gene ontology of genes correlating with response

Term	Genes	P-Value	Fisher Exacttest
homeobox	6	1,7E-3	2,3E-4
nucleus	29	1,1E-3	5,8E-4
HOX	6	3,5E-3	5,9E-4
transcription factor activity	12	3,0E-3	1,1E-3
negative regulation of signal transduction	5	1,6E-2	2,9E-3
apoptosis	6	1,4E-2	3,2E-3
chordate embryonic development	6	1,4E-2	3,4E-3
protein domain specific binding	6	1,5E-2	3,5E-3
embryonic development ending in birth or egg hatching	6	1,5E-2	3,5E-3
nuclear lumen	11	1,0E-2	3,9E-3
RNA binding	9	1,3E-2	4,3E-3
negative regulation of cell communication	5	2,3E-2	4,8E-3
nucleolus	7	1,8E-2	5,4E-3
transcription regulator activity	14	1,2E-2	5,7E-3
pattern specification process	5	2,9E-2	6,5E-3
intracellular organelle lumen	12	1,5E-2	6,6E-3
identical protein binding	8	2,1E-2	7,2E-3
differentiation	6	2,9E-2	8,0E-3
acetylation	18	1,6E-2	8,4E-3
embryonic morphogenesis	5	4,5E-2	1,1E-2
sequence-specific DNA binding	7	4,9E-2	1,8E-2
DNA binding	17	4,0E-2	2,3E-2

In the dataset of the positive response genes a significant enrichment of genes belonging to the GO group of “homeobox” genes could be identified. Also genes belonging to the functional GO terms “DNA binding” and “RNA binding” were enriched in the highly expressed genes associated with response patients. Of special interest is the fact that we observed enrichment for the GO group of “differentiation” as well as “apoptosis” underlining the role of pro-apoptotic genes, which seem to decrease the apoptotic threshold. The enrichment analysis of gene sets also identified genes associated with GO categories involving “embryonic morphogenesis” and “embryonic development”.

Tab.22: Gene ontology of genes correlating with no response

Term	Count	P-Value	Fisher Exacttest
immune system development	9	8,7E-4	1,9E-4
diseasemutation	23	1,3E-3	6,1E-4
hemopoiesis	8	1,6E-3	3,3E-4
hemopoietic or lymphoid organ development	8	2,8E-3	6,3E-4
homeostasis of number of cells	5	6,0E-3	8,2E-4
repressor	9	1,1E-2	3,5E-3
cytoplasmicmembrane-boundedvesicle	10	1,1E-2	4,1E-3
transcriptionregulation	24	1,2E-2	6,6E-3
cell activation	7	1,8E-2	5,1E-3
homeostaticprocess	12	2,0E-2	8,6E-3
kinase	11	2,1E-2	8,7E-3
cellmotion	9	2,2E-2	8,0E-3
leukocyte transendothelial migration	5	2,8E-2	6,2E-3
cytoplasmicvesicle	10	2,8E-2	1,2E-2
skeletalsystemdevelopment	7	2,8E-2	8,9E-3
regulationofapoptosis	12	3,1E-2	1,4E-2
regulation of leukocyte activation	5	3,2E-2	7,4E-3
secretory granule	5	3,3E-2	7,6E-3
regulation of cellactivation	5	3,8E-2	9,2E-3
transcriptionfactor activity	14	3,9E-2	2,0E-2
atp-binding	16	4,1E-2	2,2E-2
natural killer cell mediated cytotoxicity	5	4,1E-2	1,0E-2
nucleotide-binding	19	4,2E-2	2,5E-2
negative regulation of apoptosis	7	4,4E-2	1,5E-2
positive regulation of macromolecule metabolic process	12	4,5E-2	2,2E-2

In addition, in the no response group there was an enrichment of genes associated with the immune biology, such as genes belonging to the functional GO groups of “immune system development”, “hematopoietic or lymphoid organ development“, “leukocyte transendothelial migration“, “natural killer cell mediated cytotoxicity“ and “regulation of leukocyte activation”. Genes underscoring the high proliferation rate or maybe the more aggressive forms of

leukemia are represented by an enrichment of GO groups like “cell motion”, “cell activation”, “kinase”, “homeostasis of number of cells“, “ATP-binding”. In contrast to the genes associated with the response group, we also found an enrichment of genes negatively influencing the apoptosis such as the GO class “negative regulation of apoptosis”.

3.2.5 Pathway class comparison:

By applying a two sample t-Test a total of 292 gene sets were investigated. By using a LS/KS permutation test we detected 48 gene sets that have more genes differently expressed among the phenotype classes than expected by chance. Out of the 48 gene sets 14 were refined under the assumption that both tests used had p-value<0.05. This step allowed to further examine the biology underlying the respective response signatures, especially an enrichment of specific pathways pointing in more detail to the underlying functional deregulation in the respective leukemia subgroups.

Tab.23: Pathways that provide a significant difference between response and no response; LS/KS= least squares/Kolmogorov-Smirnov

Pathway description	Number of genes	LS permutation	KS permutation
Segmentation Clock	14	0.00003	0.00002
ADP-Ribosylation Factor	30	0.00014	0.03561
Generation of amyloid b-peptide by PS1	7	0.00085	0.00291
Bystander B Cell Activation	20	0.001	0.00366
Antigen Dependent B Cell Activation	22	0.00182	0.01461
Presenilin action in Notch and Wnt signaling	20	0.00204	0.0059
Proteolysis and Signaling Pathway of Notch	11	0.00501	0.0291
ALK in cardiac myocytes	48	0.0057	0.00164
Deregulation of CDK5 in Alzheimers Disease	9	0.0063	0.01916
The Role of Eosinophils in the Chemokine Network of Allergy	12	0.00732	0.00012
Small Leucine-rich Proteoglycan (SLRP) molecules	6	0.00864	0.02269
Th1/Th2 Differentiation	29	0.02128	0.03002
TPO Signaling Pathway	46	0.03392	0.02047
Cytokines and Inflammatory Response	26	0.04625	0.02668

Among the 14 pathways an obvious correlation with B-cell activation representing the immune mechanism via the “bystander B-cell activation” as well as the “antigen dependent B-cell activation” and “role of eosinophils in the chemokine network of allergy” pathway was seen. Quite interesting was the role of the bystander B-cell activation pathway, which is leading to B-cell apoptosis suggesting a repressive influence of the potential immune response to the leukemia cells. In addition three pathways interacting with the NOTCH pathway like “segmentation clock”, “presenilin action in Notch and Wnt signaling” and “proteolysis and signaling pathway of Notch” were found enriched. The role of the Notch pathway in AML has just recently been described [131, 154].

3.3 RT-PCR-Analysis:

For the validation of the microarray based gene expression analysis two selected genes were investigated by qRT-PCR analysis of 14 patients (6 responders and 8 no responders). After primer evaluation by regular PCR, a standard curve for each gene was executed. As already mentioned above, for the RT-PCR analysis the sequence detection system software V 2.3 as well as the 7900HT real time PCR Fast System were applied.

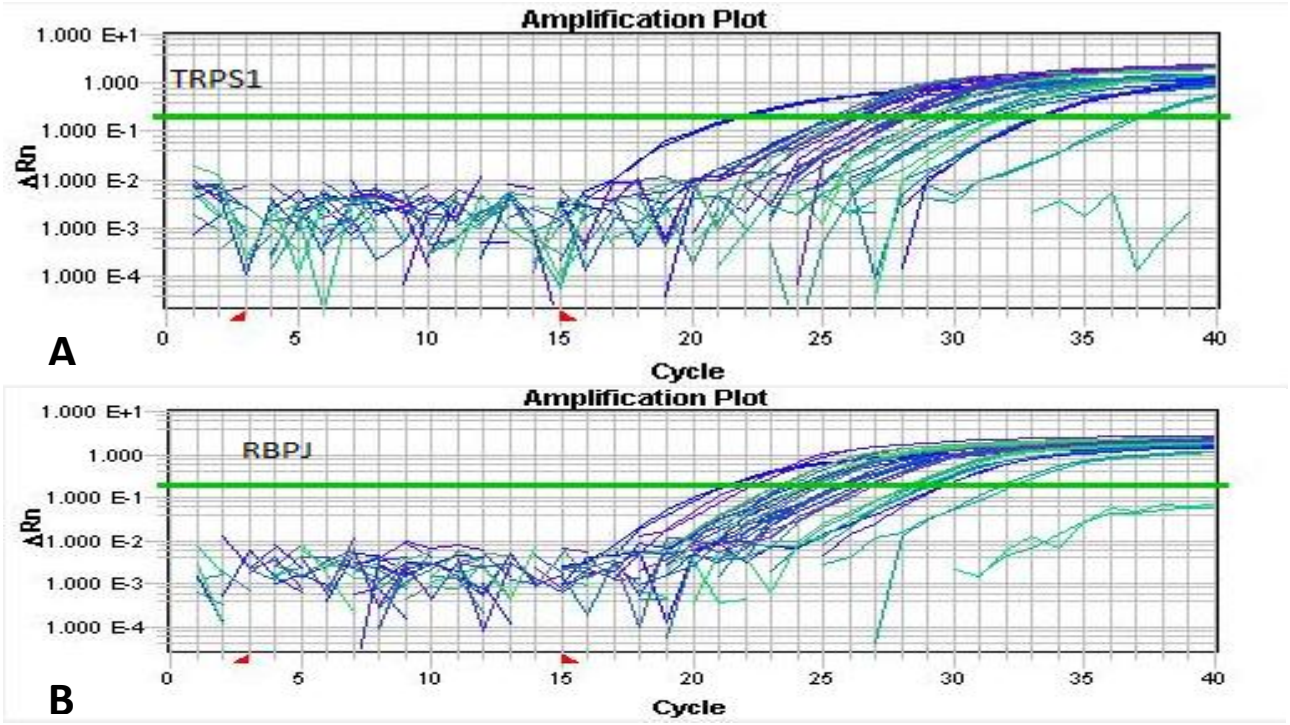

Fig.11: Amplification plots for each gene (A) *TRPS1*= trichorhinophalangeal syndrome I (B) *RBPJ*=recombination signal binding protein; presenting the DNA molecule quantification curve. The x-axis presents the cycles and y axis delta Rn. ΔRn represents the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. During the exponential amplification phase, the sequence of the DNA target doubles every cycle. In the amplification plot of *RBPJ* the black arrow marks the Ct value of the concerning sample.

Tab.24: Comparison of normalized microarray and qRT-PCR data. Using the Pearson T-Test to see if the data are set in significant correlation only *TRPS1* and *RBPJ* had significant result.(TRPS1= trichorhinophalangeal syndrome I; RBPJ= recombination signal binding protein)

	TRPS1	
Pat	Microarray Data	qRT-PCR Data
Pat1	-0,1790581	-0,6080854
Pat2	-0,0517669	-0,3476351
Pat5	-0,9555569	0,6944169
Pat6	-0,5716696	-0,9833744
Pat7	-0,4834085	-0,3744743
Pat8	0,4414744	0,2203383
Pat9	1,790651	1,822537
Pat10	0,02696463	0,7247449
Pat11	0,5606059	1,127621
Pat12	1,022821	0,6963972
Pat13	0,6475553	0,09815449
Pat14	-0,3077669	-1,072978
Pat15	0,4174914	-0,9728668
Pat16	0,02925011	-1,024796
	Pearson T-Test p=0.03	

	RBPJ	
Pat	Microarray Data	qRT-PCR Data
Pat1	-1,21504133	-1,6135151
Pat2	-0,41303937	-1,05855769
Pat5	-0,43474214	-0,64226798
Pat6	0,14569266	-1,28083875
Pat7	-0,50006835	-1,86201524
Pat8	-0,95727079	-0,37845838
Pat9	0,56733687	1,32331739
Pat10	0,17615683	0,37274177
Pat11	1,07058604	1,59137848
Pat12	0,52256186	0,04341024
Pat13	0,87484057	1,45278261
Pat14	0,27110655	1,83869165
Pat15	0,33991892	2,4750113
Pat16	-0,56014745	-2,26168032
	Perason T-Test p=0.002	

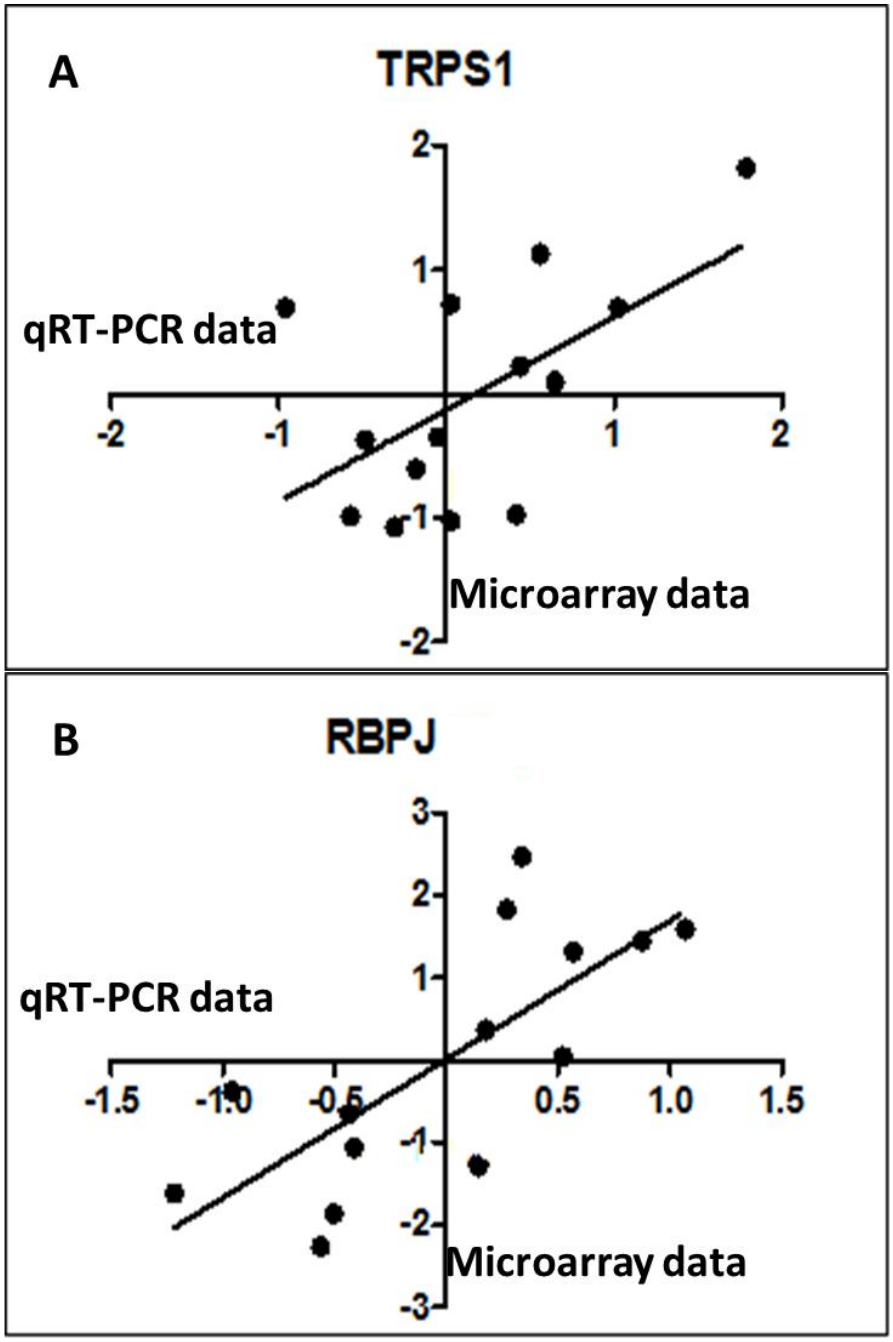

Fig.12: XY plot of the normalized and log2 transformed GEP= gene expression profiling and qRT-PCR analysis results for each gene The analysis comparing the microarray based GEP findings (y-axis) with qRT-PCR (x-axis) data did show a significant correlation of the investigated genes (A) *TRPS1*=trichorhinophalangeal syndrome I (B) *RBPJ*= recombination signal binding protein

4 Discussion

4.1 Discussion of the DAC treatment and treatment results:

Most patients diagnosed with AML are older than 65 years and therefore many of them are not eligible for an intensive therapy regimen due to comorbidities. So far, the only therapeutically option for this kind of patients was LDAC, best supportive care with hydroxyurea or just recently added, AZA. The success of LDAC has been limited however AZA as an epigenetic therapeutic seems to show promising results for high risk MDS and AML patients with a 2 year overall survival of up to 60% [77], this option is however unfortunately not approved in Europe. Based on these findings epigenetic therapy seems to open a new therapeutic strategy in myeloid malignancies. Decitabine shows similar results in high risk MDS or AML as AZA and is therefore of special interest [158].
DAC induces DNA hypomethylation in vitro and in vivo and re-establishes silenced gene expression of tumor suppressor genes and down regulates oncogenes [93, 121].

The hypermethylation of promoter DNA appears to be a trigger for attracting histone deacetylases and histone methylases and modifying histones into a down regulated chromatin state [139, 140]. There seems to be a feedback loop between DNA methylation and histone methylation [169, 170]. But in cancer cells this feedback mechanism is disturbed by DAC caused by its hypomethylation and restoring the silenced histone code at tumor suppressor loci [236]. Global hypomethylation induced by DAC therapy was detected in early trials and detailed in recent publications [49, 121].

The first clinical application of DAC within a trial, used as a single agent, was in a phase-I study in refractory or relapsed pediatric acute leukemia. The investigators used a dose schedule of 1500 to 2500 mg/m² resulting in a significant reduction of leukemic blasts [193]. Momparler and colleagues continued this study by treating 27 children diagnosed with acute leukemia with a continuous infusion of DAC over 36-60 hours. An overall response of 37% was achieved with 22% of the patients reaching a complete remission [168]. Interestingly, they found an inhibition of 70% of DNA methylation, but no signs of blast maturation. Despite these clinical

results the further usage of DAC at high dose was not pursued based on the high degree of hematological toxicity with a delay and prolongation of myelosuppression [121]. Therefore, it took almost a decade until the first phase I/II trial investigating the clinical activity of low dose DAC showed that in elderly AML/MDS patients receiving 15 to 30mg/m² for MDS and 30 to 90mg/m² for AML (given 3 times daily for 3 days) a 15% CR and 30% PR rate can be achieved, which results in an overall response rate of 45% after a median of two courses. In responding patients the median survival time was 19 weeks [184]. This study not only showed a reduction of peripheral leukemic blasts, but also an enhancement of differentiation. In the 1990s several low dose DAC phase II studies in elderly patients diagnosed with high-risk MDS provided good clinical response with comparable overall survival rates of approximately 50% and a median response duration of several months [234, 235]. The cumulative results of these trials led to a multicenter randomized phase 3 trial [132]. In total 170 patients were included to receive DAC at a dose of 15mg/m² (given as infusions 3 times a day over 3 days repeated every 6 weeks). The overall response rate in patients receiving DAC vs. best supportive care (BSC) was 30% and 7%, respectively. The median response duration was 10.3 months with a first response time of 3.3 months. A subgroup analysis indicated a great value in intermediate-2/high risk disease [132]. This study led to FDA approval for DAC in MDS patients.

In a subsequent phase II multicenter study (from which the analyzed patient samples were derived for this thesis) Lubbert et al treated elderly AML patients with DAC as a front line treatment given at a dose of 135 mg/m² every 6 weeks and for 100 patients DAC was given in combination with all-trans retinoic acid (ATRA) [159]. It could be shown that DAC is well tolerated by elderly patients and even more interestingly the response rate and overall survival were not negatively influenced by poor-risk cytogenetics or prior myelodysplastic syndrome. A similar first line treatment study applying however a lower dose of DAC (20mg/m² DAC monotherapy for 5 consecutive days in 4 week cycles) achieved a complete response rate of 24% [35]. These findings led to a phase III multicenter trial comparing DAC 20mg/m² given 5 days straight in 4 week cycles with patients choice supported by clinicians, either BSC or low dose cytarabine in elderly AML patients. DAC treatment resulted in a significantly increased response rate of 18% vs. 8% for BSC/LDAC and also an increased overall survival 7.7 months vs. 5 months [133].

However, in neither of these trials a statistical significant improved survival could be shown on primary analysis, but in an unintended analysis by Kantarijan et al (after 92% of the patients had died in the trial) the OS of patients treated with DAC was significantly prolonged (p=0.037) leading to an approval by the EMA in the fall of 2012, whereas DAC failed the FDA assessment. In contrast, AZA showed significance in prolonging OS compared to LDAC or BSC [76, 77]. Nevertheless, a comparison between these two epigenetic agents is quite complicated due to several reasons. First, there usually more cycles of AZA administered than of DAC, as there is no standard on how many cycles of DAC a patient should receive. It is often suggested that at least six cycles of treatment should be administered before declaring a patient as resistant [71], but several studies (including our investigation) have shown that around 80-90% of the responses occur within the first two cycles [158, 214]. Second, there is also a difference of the effect depending on the bone marrow blast percentage, AZA shows an increased response rate compared to LDAC in AML with 21-30% blasts [77] whereas DAC does not show any advantage in these patients compared to LDAC but is superior in patients with >30% blasts [133].The former argument may be resolved based on a phase II trial showing an even better CR rate of 47% and an overall response rate of 64% after a median of three cycles with a intensified i.v. DAC 20mg/m² application over 10 days with a well- tolerated toxicity. A CR was reached in 52% of patients with a normal karyotype and in 50% for those with a complex karyotype [14]. Based on these clinical results a randomized phase III trial with the 10-day application of DAC should be further followed up. Furthermore, there have been no randomized trials investigating AZA or DAC in comparison to more intensive therapeutic approaches like the ‘3+7 regime´. Based on a retrospective analysis in newly diagnosed AML older than 65 years treated with either a hypomethylating therapy (DAC or AZA) or an intensive chemotherapy, DAC and AZA resulted in a lower CR rate, however DAC and AZA did not seem to be adverse with regard to OS in relation to an intensive chemotherapy approach [188]. Accordingly, it seems that DAC and AZA may rival more intensive therapy for elderly patients, and in summary additional studies directly comparing DAC vs AZA and DAC/AZA vs intensive chemotherapy are warranted.

Finally, there is still a high percentage of patients that are non-responders and show resistance to DAC. These mechanisms of resistance are poorly understood, and therefore it remains

essential to establish valid clinical and biological predictors of response to further improve patient management, i.e. to better guide epigenetic treatment approaches with e.g. DAC.

4.2 Previously reported markers/predictors associated with DAC response:

Clinical variables: In a time where targeted therapy gains more and more momentum and has already been established in some types of cancer like CML, ALL, lymphomas or in several solid tumors, it is more important than ever to find precise biological targets or markers for novel treatment approaches to avoid toxic chemotherapy. So far there is only limited data of clinical pre-treatment parameters that allow a prediction of therapeutic outcome for DAC [71]. Jabbour and colleagues provided that aberrations of chromosome 5 and 7, low hemoglobin and platelets level were independently associated with a decreased OS in MDS patients treated with DAC coming to the conclusion that factors for adverse outcome retain their value in DAC treated MDS patients [122]. Lübbert et al summoned up older age, low platelets, high LDH levels and poor performance status as adverse outcome factors for DAC therapy. However there was no control arm in this study for further evaluation. In our cohort we found a high bone marrow blast count (>50%) as a significant factor for poor outcome, but not for response. This may indicate that the remission period may not last as long in highly proliferative/aggressive/advanced disease as similarly seen with intensive chemotherapy [103]. On the other hand, Kantarjian et al demonstrated that there has to be at least a blast count of 30% for DAC being superior compared to LDAC, making the suggestion that DAC seems to affect the leukemia cells not just only on the epigenetic basis but also may function as cytostatic drug. However the exact mechanism remains unknown and is a high anticipated goal for further studies.

Genomic markers: DNMT3A is taking an essential role in physiological and aberrant epigenetic signaling and mutations are found in 4% to 22% of adult AML patients [1]. In a small analysis it was shown that 6 out of 8 patients with a *DNMT3A* mutation achieved CR but only 13 out of 38 with a *DNMT3A* wild-type [165]. These results are not in line with our findings where 2 out of 6 *DNMT3A* mutated cases responded and 14 out of 29 *DNMT3A* wild type cases. However, the DAC treatment between the trials was different with regard to total dosage, administration

and duration of cycle. As mentioned above a total dosage of 135mg/m² over 3 days (15mg/m² per application) within 6 week cycles was the treatment schedule for our patients, whereas Metzeler et al gave 20mg/m² over 10 days leading to total dosage of 200mg/m² per cycle with a cycle duration of 4 weeks. Thus, it can be suggested that AMLs compromising a *DNMT3A* mutation may benefit of a DAC treatment with a prolonged administration and shorter cycles. However, it is not possible to draw a conclusion whether it is the higher total dosage or the prolonged administration over 10 days that leads to a better outcome. Therefore, further clinical trials comparing different treatment schedule are required for evaluation. Although DAC functions as a hypomethylating agent it seems to be challenging to provide consistent results demonstrating correlation between response to DAC and prior/post treatment DNA methylation changes.

Epigenetic markers: Yan et al detected in AML patients, after DAC treatment, that hypomethylation predominantly occurs in CpG islands or CpG associated regions [242]. Upon DAC treatment the expression of 81 of 22,000 genes was induced and 96 genes were down-regulated in AML cell line OCI-AML2 [218]. For example, DAC induced the expression of the cell cycle inhibitor gene *CDKN1A* that encodes for p21, which can lead to a proliferation arrest in AML cells. The expression of p21 however was autonomous of the gene's promoter methylation status. Instead it was mediated via the re-expression through the tumor suppressor TP73, hypermethylated promoter region in AML cell lines and an upstream regulator of p21 [218]. The epigenetic regulation of tumor suppressor gene *CDKN2B* (*p15*) has also been under investigation for several years now. But the results are controversial concerning the association of methylation/demethylation with clinical outcome. Multiple studies documented that partial demethylation and reconstitution of regular p15 expression after DAC treatment correlated with clinical response in MDS and leukemia patients [48, 91, 102]. However, it has to be mentioned that two of these studies used valproic acid (VPA) histone deacetylase inhibitors in addition to DAC [91, 102]. In another investigation noticing hypomethylation of p15 in hematological malignancies following DAC therapy no correlation between the methylation status prior or after therapy and response was observed [118]. Another and quite interesting target gene which seems to be activated by DAC is p53-inducible ribonucleotidereductase (p53R2/RRM2B) in a hypomethylation-independent manner [248].

Another study in MDS patients treated with DAC failed to detect any correlation between the pretreatment methylation status of 10 selected genes (none of these genes were found in our analyses) and clinical response to DAC, but the investigators were able to show a significant association between clinical response and a reduced methylation status over time [207]. Klco and colleagues observed in a cell culture study that long exposure to DAC in clinically relevant concentrations unfold in methylation and expression changes and finally in differentiation [136].The results of methylation and expression changes relied rather on the intrinsic properties of the primary cells than of DAC treatment.

In summary, the diverse findings regarding epigenetic markers may rely on the proclivity of several investigators to explore a variety of genes [71], technical limitations as well as the usage of different sample sources (e.g. bone marrow vs. non fractionated blood samples with no enrichment of leukemic blasts). Additionally, the lack of consistent association between epigenetic patterns at diagnosis, DNA hypomethylation induction and therapeutic response might question a precise *in vivo* mechanism of DAC since it seems to be more complex than so far recognized [119], although so far most studies were performed on limited cases and thus the complexity might not have been well captured yet.

4.3 Method:

Acute myeloid leukemia shows a high variability on the clinical side as well as the biological behavior.

Therefore, a scientific research method that is able to give us a reliable broad overview of the disease is required for a better understanding of its biology. Gen-expression analysis by microarray-based analysis compromises such characteristics and is therefore a frequently and well-established method for experimental clinical research. By applying this method we were able to identify several genes that correlated either with response or resistance to DAC in a cohort of 46 AML patients. Based on these results and by applying a supervised gene analysis it seems possible to draw certain conclusions of the different biology between these two groups and which may define specific phenotypes

However, microarray-based GEP is not the only method to investigate gene expression. For example the reverse transcription polymerase chain reaction (RT-PCR) a method that is able to qualitatively detect gene expression as well, however it does not provide the same quality of results as GEP and does also provide only results for a single gene. In addition, while being much cheaper it does not offer to screen over 25000 genes in a large patient cohort at a time and is therefore by far not as efficient as GEP. The *real-time or quantitative* RT-PCR maybe more sensitive and specific as conventional RT-PCR and GEP but as like conventional RT-PCR it is only capable to provide results of one gene at a time. A comparable and competitive method to GEP is the serial analysis of gene expression (SAGE). SAGE disposes a high sensitivity and specificity and through this method it is feasible to identify unknown genes in contrast to GEP. But due to high cost and expenditure of time the SAGE method is not practical for larger patient cohorts [243]. Currently, the method with the most promising expectations is next generation sequencing (NGS). NGS combines DNA/RNA sequencing on high level and also allows the global gene expression analysis. With the aid of NGS it is achievable to sequence an entire genome just within a few days [228], but of course these high power scientific operations entail high costs and by the time of experimental analysis only a few research cancer centers worldwide were capable to operate it for commercial use, while it today has become widely available and can also be performed via our Genomics Core Facility.

In summary, the GEP technology provides currently among the established and available methods the best option for gene expression analysis of large patient cohort regarding cost-efficiency and expenditure of time. For a scientific experimental method it is crucial that the results of different studies are comparable. Due to well established quality standards for the production as well as hybridization of DNA microarray and an emergence of integrative statistical algorithms even a comparability of datasets evolving from different microarray analysis can been achieved.

These contributions are essential for the value of microarray-based GEP results as former normalization errors of the fluorescence ratio intensity measurements can be avoided [187]. Interestingly, the sample preparation as well as the sample storage does not seem to have a high influence on the robustness of diagnostic gene expression signatures in leukemia [138]. Furthermore, it could be shown that results of different microarray platforms, for example

Affymetrix GeneChips in comparison to cDNA-Microarrays, can provide equivalent data. Ruecker and colleagues identified that myeloid cells generate a steady gene signature independent of microarray platform and laboratory facility [194]. Therefore, it is no surprise that different study groups provided concordant results regarding specific gene signature determining cytogenetic AML subgroups [21, 222].

The gene expression analysis helps to find new biological characteristics of certain malignant diseases and especially for myeloid diseases, where it is quite simple to collect sufficient tumor material. Due to the lack of reliable biomarkers for DAC response and due to the knowledge that no other prior investigation has been executed using genome wide GEP for the identification of molecular mechanism that may have an influence on the DAC effect, we chose this method to evaluate DAC response associated features. Prior studies have primarily been focused on the methylation status and the accompanied expression status and only a few genes have been investigated [45, 207]. Therefore, a genome-wide GEP analysis of over 25,000 genes in AML patients receiving DAC addressed this challenge in a new way and generated results that provide new insight into the therapeutic mechanisms of DAC.

For the validation of our results we chose 5 different genes that were significantly differently expressed between the response and no response group and assessed with real time RT-PCR. As mentioned above the real time RT-PCR options as good method for a respective validation due to its high sensitivity and specificity and is hence a reliable method for the gene expression analysis of single genes.

4.4 Discussion of class-comparison analysis results:

Epigenetic therapy is just in its early stages and so far there is a lack for clinical and molecular markers that may predict response in patients with AML. Therefore, it is essential to detect biological characteristics to apply the proper treatment and to avoid ineffective toxic therapy, and we analyzed the GEP of AML DAC treated patients to find potential biological characteristics that (i) may help to predict response and (ii) may help to establish a better understanding of the epigenetic mechanism in AML. In the gene expression class comparison

analysis 301 genes were detected that were associated with either DAC response or resistant disease.

4.4.1 Up-regulated genes in responders:

For the response group 124 significantly higher expressed genes were identified involving genes like: *ASCL2, INPP5D, SOX4, MSI2, SLC24A3*, *PRAME, HOXB9, HOXB7, HOXA4, TNSFS9, MSX2* and *MLL.* Several of the genes like *SOX4, HOXA4, MLL* and *MSI2* have been mentioned before in AML studies and interestingly have been put in correlation with adverse outcome [21, 135].

SOX4, down-regulates the essential transcription factor for a normal hematopoiesis PU.1 and GATA3 the master for T-helper cell 2 differentiation. By interacting with the cAMP response element-binding protein (CREB) a nuclear transcription factor highly expressed in neoplastic hematopoiesis, *SOX4* contributes to increased proliferation of hematopoetic progenitor cells[135] [7, 198]. In ALL *SOX4* enables oncogenic survival signaling and correlates with adverse outcome as well [190]. These data support the concept that *SOX4* seems to be an essential oncogenic driver in hematologic malignancies and has even been suggested as new potential therapeutic target [7]. Another gene like *TNSFS9* acting as T-cell inducer [240], and correlating with AML progression malignancies has also been found and therefore may underline the role of the immune response in hematologic cancers [113].

HOXA4 overexpression has been implicated with high WBC [148] and with poor outcome [21, 107], and it has been shown that the expression level is governed by the methylation state of the gene [246]. Next to *HOXA4* we found two other homeobox genes: *HOXB9* and *HOXB7. HOXB9* has been found highly expressed during oncogenic driven myelomonocytic differentiation [232] whereas *HOXB7* if overexpressed blocks differentiation in HL60 leukemia cells [223]. In addition, it has been associated with progression in multiple myeloma [215] underscoring the fact that dysregulation of homeobox genes is essential in hematologic malignancies [223].

The transcriptional co-activator *MLL* plays an important role in regulating gene expression including HOX genes like *HOXB9*, *MSX2* and *IRX-3* (all genes found with an increased expression in responders) during hematopoiesis [206]. Poppe at al proposed that the activation of genes in MLL rearranged and overexpressing leukemias are similar, leading to the assumption that a

common gene expression pattern with its transcriptional program is causing clinical similarities of MLL-rearranged and MLL overexpressing leukemias and therefore might explain the resistance to conventional chemotherapy [186]. The RNA binding protein MSI2 is expressed in HSCs (hematopoietic stem cell), targets cell cycle regulator like *RAS* and its knock down results in HSC depletion. Furthermore it is found to be overexpressed in AML and its expression level directly correlates with worse patient outcome. Due to its function for stem cell maintenance it is suggested that MSI2 overexpression is an early event of the leukemogenesis [31, 135].

Hence, it is very interesting that AML representing an expression pattern like *SOX4, TNSFS9, HOXA4, MSI2* and *MLL* seem to be responsive to DAC whereas they show resistance to conventional cytostatic therapy. Conventional chemotherapies rely on the antimetabolite effect of the drugs that often show no effect in aggressive leukemias due to certain molecular mechanism. However DAC attacks leukemia cells in a different way by attempting to change their gene expression pattern. It has been proposed before that DAC may achieve response in AML, whereas cytostatic treatment fails [159]. *SOX4* suppresses T-cell differentiation and therefore avoids a sufficient immunological response, however, DAC was shown to have the capability to induce cancer testis antigens, thereby generating an efficient immune response with tumor cell lysis by CD8+ T-lymphocytes. A similar suggestion was provided for *TNSFS9* because DAC might be also effective based on an immunomodulatory effect. It seems that the poor prognostic impact of *SOX4* and *TNFSF9* might be overcome by DAC, thereby rendering *SOX4* and TNSFS9 as positive markers for DAC response.

Aberrant *HOX* gene expression is leading to a dysregulated hematopoiesis by blocking differentiation. *HOXA4* is regulated by the polycomb group of genes (PcG) [107]. In a comparison of PcG and PcG regulated genes revealed correlations to genes involved in DNA methylation including DNMT1, which is directly inhibited by DAC, generating the suggestion that DAC may intervene in this pathway complex. Due to similar aberrant gene expression findings in *MLL*-rearranged and *MLL*- overexpressing leukemias, aberrant epigenetic changes have to be involved. Whitman et al showed that in leukemias with *MLL* partial tandem publication DAC response relies on the re-expression of the TSG *SLC5A8* [233]. These results concurrent with ours underscore that re-expression of TSGs via DAC is one of the mechanisms to attack AML by rendering it again sensible to treatment.

Similarly, self-renewal, an important feature of leukemic blasts, was shown to be regulated by *MSI2* due to transduction with the fusion protein Vp16/Meis1 [135]; interestingly, *Meis1* is also a *MLL* target gene and upregulated in MLL rearranged leukemia. Based on these interactions DAC treatment may also result in blocking self-renewal of leukemic blasts due to re-expressing genes that are down regulated among these molecular conditions. In summary, DAC therapy seems to overcome certain aggressive leukemia subtype characteristics via different anti-leukemic aspects to, reflecting, thereby rendering DAC an alternative treatment option.

Next to the upregulation of genes associated with adverse AML outcome in patients treated with conventional therapy, we also found genes that have been related with favorable outcome including *PRAME* and *INPP5D* [104, 149]. The tumor associated antigen (TAA) *PRAME* functions as a repressor of RAR signaling [65] and its expression is epigenetically regulated [109]. Just recently a study showed that in leukemia cell lines PRAME impairs the retinoic acid-regulated-cell-proliferation and differentiation, which seemed to be reversed by ATRA leading to a better outcome in patients with NPM1 mutation [25]. The effect of ATRA however does not rely on DNA methylation changes [166]. Compared with our results it seems that AML expressing PRAME shows two ways of therapeutic targeting. One based on reversing the repression of *RAR* signaling through ATRA and the other one by using PRAME epigenetic based expression and its TAA property by DNA methylation changes and immunomodulation through DAC. Taken together in these types of AML ATRA and DAC may have a synergistic effect leading to an improved response rate or outcome.

INPP5D is a negative regulator of the PI3K/AKT pathway, which is constitutively activated in 50-70% of AML [16] and AKT phosphorylation has been linked to drug resistance [98]. Expression of *INPP5D* generated downregulation of constitutively active AKT and prolonged cellular transit through the G1 phase, decreasing cell growth, and additionally loss of *INPP5D* accelerated leukemogenesis [147] pointing out its function as a fundamental TSG. The general linkage of *INPP5D* expression and superior outcome may rely on the effect of generating drug sensitivity, but under DAC treatment it may also rely on the delayed G1 phase. DNMT1 is recruited and functions during the S-phase, however it has also been shown that DNMT1 is active during the G0/G1 phase leading to the suggestion that the *INPP5D* expressing cells are more susceptible to DAC.

ASCL2 and MUM1 have not been described in the concept of AML yet, but seem to be involved in the pathogenesis of other malignancies [50, 112]. The Wnt target gene *ASCL2* is epigenetically regulated via DNA methylation and has been correlated if methylated with adverse outcome in colon cancer. However, its role in the malignant pathogenesis remains controversial [50, 128]. Finally, MUM1 previously associated with poor prognosis in multiple myeloma [112] encodes also a gene important for interferon dependent immune response, thereby further underscoring a potential immunomodulation effect of DAC.

Since DAC is an analog of the natural nucleoside 2'-deoxycytidine, it is commonly accepted that the DAC prodrug is transported into the cell by the equilibrated-nucleoside system, including sodium-nucleoside transporters like the SLC28A family. However, these transporters rely on the intra-/extracellular sodium gradient. In the responder group a significant higher expression of the sodium-/potassium-/calcium-exchanger has been detected leading to the suggestion that due to a stable sodium gradient caused be the sodium/potassium/calcium-exchanger results to a higher activity of the sodium-nucleoside transporter. A higher activity of these sodium-nucleoside transporters might automatically generate a higher intracellular concentration of DAC. The higher concentration in the cell may possibly translate into an increased response via DNA hypomethylation.

4.4.2 Up-regulated genes in non-responders:

For patients who did not respond to DAC 177 genes significantly higher expressed were found, including e.g. *IFI27, IFI44L, THBS1, FAS, MX1, TRPS1, RBPJ, ITGB2, ADAM9, NOTCH2, PDK4* and *HDAC4*.

The immune and inflammation-related genes *IFI44L* and *IFI27* might reflect a hyperstimulated, but insufficient immune system and as it has been recently shown in myelofibrosis [210]. As mentioned above DAC re-expresses cancer testis antigens leading to immune activation, however an impaired immune system may prevent response to DAC.

THBS1 plays an essential role angiogenesis inhibition and is also defined as TSG by inducing apoptosis through the caspase pathway in leukemic cell lines as well as by inducing differentiation in APL [19] [151]. Regarding the apoptotic effect of *THBS1* its importance to be expressed has been underlined by a study in malignant melanoma. This study shows that in malignant melanoma cell lines with *THBS1* silencing due to promotor hypermethylation

treatment with AZA leads to a restoration of expression and decreased growth of the tumour cells [152]. In addition, high expression of the pro-apoptotic genes *MX1* and *FAS* in the non-response group were observed as well. Notably, MX1 and FAS have been shown to be repressed by promoter hypermethylation in distinct AML subtypes and treatment with a hypomethylating agent in this case AZA was able to upregulate their expression levels [55, 72]. Therefore, in contrast to these findings high pre-treatment expression levels might indicate that in the respective AML cases deregulated promotor methylation silencing of pro-apoptotic genes might not be the prominent pathomechanism, and thus these cases might less likely benefit from hypomethylating agent like DAC.

Recently published data pointed to the impact of the NOTCH signaling pathway in AML [131, 154] where NOTCH1 is rather seen as a TSG in contrast to T-cell leukemia. However, its exact role in the complex mechanism of leukemogenesis remains crucial due to its inhibitory role in myeloid differentiation [146]. Besides *NOTCH2,* two other genes *RBPJ* and *TRPS1* involved in the NOTCH signaling complex have been found. RBPJ functions as a repressor when not bound to Notch proteins and as an activator when bound to Notch proteins. It also recruits chromatin remodeling complexes containing histone deacetylase or histone acetylase proteins, like HDAC1. HDAC1 has the ability to bind DNMT1 and purifies its methyltransferase activity from nuclear extracts [87], explaining a potential DAC treatment failure, because of the high DNMT1 activity. TRPS1 acts as suppressor of GATA regulated genes, whereas GATA functions primarily pro-leukemic [178]. Since activated NOTCH signaling seems to be a potential inhibitor pathway in AML, the suggestion can be made that AML with high NOTCH2 expression, as seen in non-responders, and increased expression of other important NOTCH pathway genes might be more aggressive. As mentioned above, this opens the possibility that the suppressive influence of NOTCH in AML is limited depending on co-factor activation and falls into the category of failed hypomethylating effect by DAC since TSG silencing is not the prominent pathomechanism.

The metallopeptidase ADAM9 interacts with SH3 domain-containing proteins, which functions as a cytoplasmatic adaptor protein to positively regulate transcriptional activity. ADAM9 activity has primary been associated with aggressive solid tumors, especially for spreading metastasis [85]. So far ADAM9 has not been mentioned in the context of AML. As it is

characteristic for cell invasion and migration in solid tumor, it may automatically influence the leukemic blast to escape from the controlled niche for further proliferation.

ITGB2 an integral cell-surface protein participating in cell-surface mediated signaling has been associated with drug resistance in CLL and multiple myeloma and associated with DAC resistance in this study [160]. In AML recently ITGB3, another member of this integrin protein family, was shown to be mandatory for leukemogenesis but non -relevant for normal hematopoiesis [167]. With the finding of high expression of ITGB2 associated with no response, a new role of the integrin cell surface protein family in hematologic malignancies has been added.

The gene *PDK4* encodes a mitochondrial protein involved in the regulation of glucose metabolism. It helps to decrease metabolism and conserves glucose generating a survival advantage compared to cells which are in high need of glucose in a proliferative stage as seen in the bone marrow. *PDK4* is induced by the CCAAT/enhancer-binding protein beta (C/EBPbeta) [6] which seem to collaborate with EVI1 induced AML and is also associated with leukemic stem cell exhaustion and *FLT3*-ITD mediated signaling [110, 230]. In this analysis *PDK4* was associated with no response to DAC, probably reflecting the association with poor outcome molecular aberrations.

Due to the interdependence of DNA methylation and histone modification the increased expression of *HDAC4* in non-responding patient samples underlines the complexity of epigenetic regulation. *HDAC4* possesses histone deacetylase activity, which generates repressed transcription when tethered to the target gene promotor. However, it does not bind directly to the DNA, but uses the transcription factors *MEFC2* and *MEF2D*. It has been shown that HDAC4 interaction with GATA1 leads to repression of the GATA1 transactivating potential and thus inhibits erythroid differentiation [229]. It also has been published that HDAC4 inhibition reduces the hypoxia-related increase of glycolysis and resistance to docetaxel chemotherapy [96]. These findings in addition to this study's results might indicate that HDAC4 may also suppress other TSG genes and that high activity of histone modulation decreases the effect of the DNA hypomethylation of DAC, even though both mechanisms seem to be linked together.

4.4.3 Discussion of Supervised Cluster Analysis:

Hypomethylating therapy with the usage of DAC or AZA has been under heavy investigation among the last years. However, so far there are not any valid predictors for outcome in AML patients receiving these agents. Based on the class comparison analysis with the 301 significant differently expressed genes between the two patient groups a supervised gene cluster analysis was performed in order to see if these genes are able to discriminate between the two groups and thus could be used to create a valid predictor for outcome. Group I reflects mainly responders (66%), and in group II 72% of non-responders are found. These results indicate that the gene expression patterns may help to create a predictor indicating outcome prior to DAC treatment. However, due to the small number of cases and a high rate of false positives of around 30% further studies are required and essential. Nevertheless, these results are the first to demonstrate the potential value of potential gene expression based DAC outcome predictor and provide a first step for the evaluation of new biological markers for DAC response.

4.5 Discussion of gene expression survival analysis:

In most clinical studies an important primary endpoint is response to therapy, which may be often reached, however, it is often failed to show a significant increased overall survival. Since the type of response has to be seen different in epigenetic therapies, because of their not primarily cytotoxic effects in leukemia, which is in contrast to general intensive therapy, a gene expression based survival analysis was performed. It is quite frequently noticed that patients receiving a remission to DAC therapy even it is just a PR, remain in remission longer than expected [121]. Therefore, an analysis was set (i) to see whether specific genes that show susceptibility to DAC also come along with an increased survival, and (ii) to specifically select genes that are associated with a good prognosis. The identified genes were compared with the 301 genes found in the class comparison analysis and 19 of them were congruent. Of these 19 genes, one correlated with response and good outcome, whereas the other 18 genes were associated with non-response and adverse outcome, and no genes where found that showed any discrepancy.

The one gene that was accompanied with response and prolonged survival was *CTBP1,* which has been described mostly as oncogene due its transcription suppression of TSGs and was associated with adverse outcome in solid tumors like breast cancer or melanoma [53, 57]. In myeloid disease *CTBP1* functions as a corepressor by interacting with EVI1 to repress anti-proliferative transforming growth factor b signaling [120]. Its influence to the DAC treatment success seems to be significant. Particularly its interaction with EVI1, which can be decreased by histone deacetylase inhibition in AML might openR further insight its potential epigenetic role.

In contrast 18 correlated with decreased outcome and non-response. Including *ADAM9* and *OLA1,* regulating oxidative stress response and often highly expressed in human malignancies [216], underscoring the potential to increase the aggressiveness in tumors or *ALCAM* a leukocyte cell adhesion molecule involved in the regulation of long-term hematopoietic stem cell engraftment and self-renewal, may explain the resistance as well as the high chance of relapse, because of the stem cell characteristics in certain leukemia blasts leading to a decreased survival. The member of the Notch signaling pathway RBPJ has already been discussed. The increased expression of TSG *MXD1* antagonizing MYC-mediated transcriptional activation [108] correlated with decreased survival. However, the most interesting finding in this analysis was that high expression of *RRM2B/p53R2*, a DNA-hypomethylation independent DAC target gene [153], was associated with adverse survival. *RRM2B/p53R2* seems to lose its anti-proliferative effect if already expressed prior to the DAC effect and further suggests that re-expression of TSGs is essential for DAC treatment success.

4.6 Discussion of gene set enrichment analysis findings:

In a following step a gene set enrichment analysis was performed to see whether certain gene ontology (GO) groups are enriched in the gene signatures associated with DAC response and non-response. This analysis may give further insight of the DAC effect in AML and point to important biological mechanism that could be further exploited to improve the DAC effect.

In the gene expression sets of DAC responsive cases the GO term of “Homeobox” could be found. Homeobox genes mainly appear in clusters A, B, C and D located on four different

chromosomes [191]. These gene clusters are pivotal for the embryogenesis and hence stem cell function, therefore it seems not to be surprising that GO groups like “embryonic morphogenesis” and “embryonic development” have been detected as well. The HOX gene group has been targeted in leukemia investigation over last couple years [2]. Since differentiation associated with the embryonic development is mainly regulated by epigenetic changes, it seems likely that these GO groups may be essential in the epigenetic deregulation of AML. In AML HOX genes are often found to be deregulated, like in MLL-translocated leukemia, which increases HOX gene transcription generating a blocked myeloid differentiation and enhanced proliferation [2]. DAC may reverse this effect by re-expression of certain TSGs that suppress HOX gene activity, suggesting that AML with an increased HOX activity are susceptible to DAC. Another reason for the DAC susceptibility might be that AML subtypes that show increased expression of genes in groups like “embryonic morphogenesis” and “embryonic development” highly rely on deregulated epigenetic changes which seem to be reversible by DAC.

Other interesting GO groups included “differentiation” as well as “apoptosis” underlining the role of pro-apoptotic genes. It is suggestive that the apoptotic threshold is increased in AML leading to DAC susceptibility by re-expression TSG that will overcome the apoptotic or differentiation threshold.

In the GSEA of the gene signature in non-responding patients mainly GO groups involved in the immune biology were seen like “immune system development”, “hematopoietic or lymphoid organ development“, “leukocyte transendothelial migration“, “natural killer cell mediated cytotoxicity“ and “regulation of leukocyte activation”. These findings point out the importance of cancer immunology. Especially in hematopoietic malignancies where immune therapy is an essential pillar and is even the only curative option, presented by bone marrow transplantation, in AML so far. However these GO group reflect a highly active intracellular biology. It is widely accepted that active immunological pathways induce either cytotoxicity for opposing cells or proliferation for the immune cell itself. Therefore, it may be possible that activation or increased gene expression of genes belonging to these GO groups in leukemic blasts leads to a proliferation and survival advantage in contrast to their normal hematopoiesis counter parts. As already discussed earlier DAC is able to re-express cancer testis antigens

generating an efficient immune response, however with an already highly active immune-system reflected by these GO groups, certain AML subtypes might not be as susceptible to DAC because of the loss of the this potential effect.

GO groups like “cell motion”, “cell activation”, “kinase”, “homeostasis of number of cells“, “ATP-binding” are representatives of a high proliferation rate and seem to reflect more aggressive subtypes of leukemia. In contrast to the response group genes negatively influencing the apoptosis are found within their regarding GO class like “negative regulation of apoptosis”. This GO group enrichment might be associated with increasing the threshold of apoptosis, thereby decreasing the effect of re-expressed pro apoptotic TSGs by DAC, which in turn might explain the resistance phenotype.

4.7 Discussion of pathway class comparison results:

The next step was two get into further functional detail of the DAC response associated gene expression signature by applying a pathway class comparison analysis.

Among the 14 significantly enriched pathways there was an obvious correlation with B-cell activation representing the immune mechanism via the “bystander B-cell activation” as well as the “antigen Dependent B-cell activation” pathway. Quite interesting is the role of the “bystander B-cell activation” pathway, which is associated with B-cell apoptosis suggesting a repressive influence of the potential immune response to the leukemia cells. An important part of this pathway is *FAS* seen highly expressed in the non-responding patient samples, providing an essential factor for taking B-cells into apoptosis. In addition, three pathways interacting with the NOTCH pathway like “segmentation clock”, “presenilin action in Notch and Wnt signaling” and “proteolysis and signaling pathway of Notch” were found. The discovery of these three pathways further points to the relevance of (potentially epigenetically) deregulated NOTCH signaling in AML, which has drawn a lot of attention recently as two studies showed a tumor suppressor function of NOTCH in AML cell lines [131, 154]. The activation of the “segmentation clock” pathway, relies on Notch1/Notch2 activation through cell-cell signaling leading to HES1 transcription. Just recently it has been provided that increased expression/activation NOTCH or HES1 seem to inhibit cellular growth in AML [131].

However, in our study increased Notch activation was indicative for treatment failure. This may suggest that Notch activation is an early event in the TSG regulation mechanism to take leukemic cells into apoptosis, but if these cells are able to adapt to this suppressive signaling they become more aggressive throughout the course of the disease. Additionally, it is presumed that the tumor suppressive signaling of Notch is quite complex and relies on co-factors. An example for this may be the “presenilin action in Notch and Wnt signaling” pathway that involves ß-catenin as a downstream target. ß-catenin functions as transcription factor activator leading to expression of oncogenes like *MYC* and *CCND1.*

4.8 Conclusion:

Epigenetic therapy seems to be a good option in the treatment of AML patients not eligible for intensive chemotherapy. So far only limited pre-treatment parameters are available that allow the prediction of therapeutic outcome following DAC therapy. By executing a GEP analysis from therapeutically naïve patient samples and by comparing findings with the clinical DAC response data it was possible to generate a gene cluster that increases the chance to predict the patient outcome to DAC treatment. Additionally several biologically interesting aspects were found, which might contribute to a better understanding of the exact molecular effects of this drug and which might help to increase its impact in AML.

Based on our and others work it can be claimed that the anti-leukemic effect of DAC is a cooperation of certain biological molecular mechanism and each seems to be essential. One aspect is the potential activation of an immunological response due to re-expression of TAA leading to T-cells associated cell toxicity. However, if certain mechanisms already suppress the immune system DAC seems to lose its impact. This is also seen if important TSG are already expressed, which does not only reflect a possible aggressive subtype, but it is also a factor that suppresses the DAC impact by taking away its targets. On the other hand AML subtypes that show an increased expression of genes reflecting epigenetic dependent cell homeostasis seem to be quite sensitive to DAC, but also the interaction of the two main epigenetic mechanisms DNA methylation and histone modification seem to be essential for DAC treatment success. This analysis opened up further discussion and future scientific aspects for the role of DAC in AML therapy and might help to optimize the epigenetic treatment success by adapting the therapy regime accordingly.

5 Summary

Introduction:

The hypomethylating agent decitabine (DAC) represents a therapeutic option for acute myeloid leukemia (AML) patients who are not eligible for an intensive treatment regime. However, there are no reliable biomarkers available yet that can predict patients who will likely benefit from this epigenetic therapy. Therefore, we executed a gene expression analysis prior to the treatment of patients with DAC in order to evaluate gene expression patterns associated with response to DAC that ultimately might be used to predict DAC outcome. Patients had been entered in a multicenter phase II trial of DAC as first-line treatment of older AML patients judged unfit for induction chemotherapy (NCT00866073).

Material and Methods:

Specimens: Blood or bone marrow AML samples were collected from elderly patient who had been entered in a multicenter phase II trial of DAC as first-line treatment of older AML patients judged unfit for induction chemotherapy (NCT00866073).

Cytogenetic and molecular genetic analyses: Samples were studied centrally by the lab of the AMLSG at the University Hospital of Ulm by conventional cytogenetic analysis and reverse transcriptase polymerase chain reaction (RT-PCR) for the recurring gene fusions resulting from t(8;21)(q22;q22), inv16(p13q22)/t(16;16)(p13;q22), t(15;17)(q22;q12-21), and t(9;11)(p22;q23), and all diagnostic samples were screened for mutations in *FLT3* (FMS like tyrosine kinase 3), *NPM1* (nucleophosmin), *CEBPA* (enhancer binding protein alpha) and *DNMT3a* (DNA methyltranferase).

Gene expression profiling: A gene expression profiling analysis in clinically well annotated specimens (n=36) was executed using Affymetrix microarrays (Human Genome U133 Plus 2.0 Arrays; 3`IVT Express Kit) according to the manufacturer's recommendations (Affymetrix, Santa Clara, CA). Filtering the fluorescence ratios JustRMA algorithm was applied. We investigated significant different gene expression patterns between responders and no responders DAC treatment.

qRT-PCR: For Validation of the gene expression profiling results, we performed a quantitative RT-PCR analysis of randomly picked genes using the TaqMan 7900HT.

Statistical Analysis: Supervised analyses including gene and pathway class comparison were carried out using BRB-Array Tools Version 4.3.0 Beta_3, and further statistical calculations were performed using SPSS, version 19 and Graph Prad-Prism version 5.

Results and conclusion:

While the study cohort comprised a heterogeneous group of AML patients, a class comparison analysis nevertheless could reveal a DAC response associated gene pattern comprising 301 genes at a significance level of $p<0.05$. This signature was enriched for genes belonging to pathways that are essential in immune response and tumor suppressor function such as the Notch pathway. While genes associated with DAC response including: *ASCL2, INPP5D, MSI2, SLC24A3*, *PRAME, TNSFS9, MLL* and several homoebox genes might be used as novel biomarkers, along genes that were significantly higher expressed in patients who did not respond to DAC treatment, such as *IFI27, IFI44L reflecting an insufficient immune system, several TSG like THBS1, FAS, MX1 or genes involved in the Notch pathway including TRPS1, RBPJ, NOTCH2*. The clinical implementation of findings warrants additional studies. In the future integrative analysis also taking epigenetic changes into account might further improve the value of transcriptomic based predictors and lead the way to improved patient management.

6 References

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7 Supplement:

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