Characterization of relapsed Acute Myeloid Leukemia

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Chapter 1

Introduction

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is diagnosed when the bone marrow of patients contains an abnormal high percentage of immature blood cells of the myeloid lineage (blasts). In AML these leukemic blasts are by definition increased above 30% in the bone marrow of pediatric patients and above 20% in adult patients[1], which interferes with normal hematopoiesis.

In children (aged ≤18 years), approximately 30% of cancers are blood cancers (leukemias), the most common type of cancer and around 15% of these patients suffer from AML[2]. The incidence of AML in young children under the age of 4 is around 1.6 in 100.000 children. Until the age of 5-9 years old the incidence declines, but then gradually increases to a maximum of approximately 23 per 100.000 individuals at age 80-84. It is estimated that 1 in 254 people will be diagnosed with AML during their lifetime. With a median age of approximately 66 years, AML is the most common type of leukemia in individuals over 65 years. Death rates for AML increase with age: approximately 2% of deaths occur in children and young adults (<20 years), while nearly 45% of deaths occur at ages above 75 years old[2,3]. In the Netherlands, it is estimated that approximately 610 individuals (~25 cases are children) will be diagnosed with AML each year and approximately 475 (8 children) will die from the disease in that same year. As most high income countries face aging populations, these figures will increase significantly in the upcoming decades.
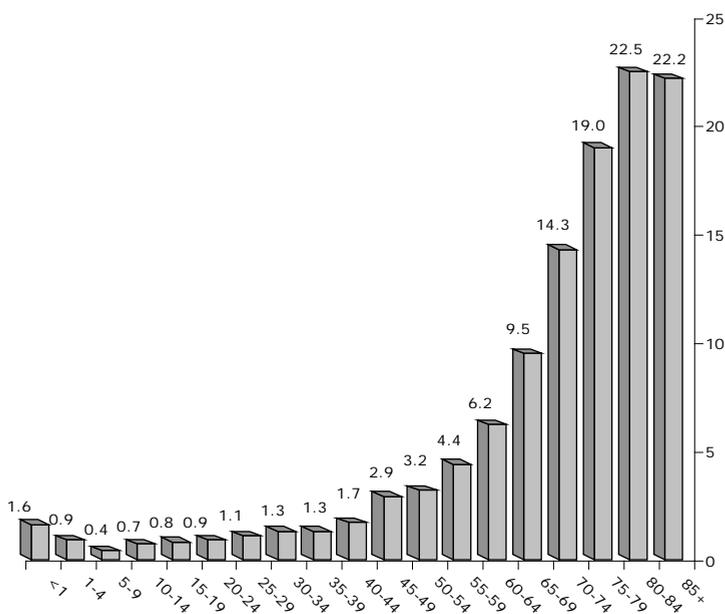
Figure 1. Incidence of new AML cases per year per 100.000 individuals per age group (5y age intervals) in the United States[2].

Genetic aberrations relevant to AML

In general, cancer is considered a genetic disorder in which mutations accumulate and cause unregulated cell growth. This model was applied to AML by Kelly and Gilliland, who proposed a "two hit" model for leukemogenesis[4]. In this model, a first type of mutation (type-I) confers a proliferative advantage to myeloid precursor cells and a

second type of mutation (type-II) impairs differentiation and enhances survival of these cells. Most studied genetic lesions that constitute these two types of mutations are for type-I, gene mutations, that result in impaired or changed function or activation of proteins and for type-II, chromosomal aberrancies that frequently involve transcription factors and impair or change protein function or result in fusion proteins with abnormal functions.

Table 1. Recurrent chromosomal aberrations in pediatric and adult AML

Cytogenetic aberration	Frequency in children	Frequency in adults	Genes involved	Subgroup
Favorable	25			
•t(8;21)(q22;q22)	12	6	*ETO-AML1*	M2/chloroma
•Inv(16)(p13;q22)/ t(16;16)(p13;q22)	6	5	*MYH11-CBFβ*	M4
•t(15;17)(q22;q21)	7	10	*PML-RARα*	M3
Intermediate	50			
Chromosomal aberrations not classified as favorable or unfavorable (mostly normal karyotype)				
Unfavorable	25			
•-7/7q-	3	5		Not specific
•-5q/5q-	1	7		Not specific
•inv(3)(q21q26.2)/ t(3;3)(q21;q26.2)	<1	1	*RPN1-MECOM (EVI1/MDS1/EAP)*	Not specific
•t(4;11)(q21;q23)	<1	<1	*MLL-MLLT2(AF4)*	Eosinophilia
•t(5;11)(q35;p15.5)	1	1	*NUP98-NSD1*	Not specific
•t(6;9)(p23;q34)	1	<1	*DEK-NUP214*	M2,M4
•t(6;11)(q27;q23)			*MLL-MLLT4(AF6)*	
•t(7;12)(q36;p13)	1	0	*ETV6(TEL)- HLXB9(MNX1)*	Infants
•t(9;22)(q34;q11.2)	<1	<2	*BCR-ABL1*	
•t(10;11)(p12;q23)	3	1	*MLL-MLLT10 (AF10)*	M5/Infants
•Complex (>3 aberrations)	14	11	Variable, e.g. *TP53, MSH2*	

Frequencies are given as % of total [5–11]

A number of recurrent chromosomal (cytogenetic) aberrations and gene mutations have been observed in AML[12,13]. Approximately 20% of the pediatric[6,8,10] and 45% of adult AML[14] patients harbor no detectable chromosomal aberrancies (cytogenetically normal; CN-AML). The recurrent chromosomal aberrancies that are found in the remainder of patients are listed in Table 1. The frequency of several of these aberrations, for example the prevalence of CN-AML cytogenetics, is age dependent. Recurrent chromosomal abnormalities have a strong prognostic impact (Table 1). For example, in both pediatric and adult AML t(15;17)(q22;q21), t(8;21)(q22;q22) and inv(16)(p13;q22) render a favorable prognosis. In contrast, e.g. deletions of chromosome 5 or 7 are associated with dismal outcome.

Next to chromosomal aberrancies also small deletions, insertions and point mutations cause aberrations in genes that are characteristic for AML or AML subgroups (Table 2). Some of these gene mutations occur at high frequencies and are of strong prognostic relevance, for example *FLT3/ITD* mutations (~10% of cases) or *NPM1* mutations (~30% of cases) that occur predominantly in normal karyotype AML. Of other mutations that occur at a lower frequency, such as *KIT* or *RAS* mutations that occur predominantly in CBF-AML, the prognostic relevance remains to be determined.

Table 2. Established or novel recurrent gene mutations and epigenetic aberrations in pediatric and adult AML					
Class	Gene	Incidence children (%)	Incidence adults (%)	Biological effect	Subgroup
Type I	*FLT3*	18	25	Proliferation	CN-AML
	RAS	20	6	Proliferation	CBF-AML
	KIT	5	5	Proliferation	CBF-AML
	JAK2	rare	rare	Proliferation	CBF-AML
	PTPN11	2-5	0-6	*RAS* activation	FAB-M5
Type II	*NPM1*	8	21	Impaired differentiation	CN-AML
	CEBPA	6	22	Impaired differentiation	
	PU1	-	0-7	Impaired differentiation	
	MLL-PTD	rare	3-11		
Type III	*DNMT3A*	rare	15-23	Aberrant DNA methylation	CN-AML
	IDH1/2	rare	7-19		FAB-M2
	TET2	rare	7-24.5		CN-AML
	ASXL1	rare	5-17	Unknown, chromatin remodeling?	CN-AML Trisomy 8
Type IV	*WT1*	10	rare		
	TP53		15	DNA damage response	

Frequencies are given as % of the total AML patient groups [13,15–20]

The identification of recurrent gene mutations in AML may be valuable for improving the prognostication of intermediate risk group patients in which cytogenetics lacks strong prognostic relevance. Established examples are *FLT3/ITD* mutations (unfavorable)[21] and *NPM1* or bi-allelic *CEBPA* mutations (favorable)[22–25]. Although associated with outcome in adult AML, the relevance as independent prognostic factors in children of other gene aberrations that occur less frequent, such as *WT1*, *KIT*, *RAS*, TP53, *IDH1/2, TET2* mutations, is subject of debate.

Recent studies that integrate clinical with molecular data[12,16] show that the genetics of AML is highly complex as the observed combinations of genetic aberrations are very heterogeneous within and between patients.

Following the discovery of recurrent molecular aberrations that involve genes that are implicated in epigenetic regulation, Dombret et al.[26] proposed a third class of mutations (type-III) that consists of genetic lesions in genes that are implicated in epigenetic modification, e.g. *DMT3A*, *ASXL1*, *TET2*, *IDH1/2* or *EZH2*[27]. A fourth class of mutations could consist of tumor suppressor type of aberrations or genetic aberrations that are associated with poor treatment response and worse patient outcome[26].

Besides molecular aberrations at the DNA level, aberrancies at other biological levels may also be associated with leukemogenesis or prognosis and may be used in classification or risk stratification of AML[9,11,28]. For example, aberrantly high expression of individual genes such as *WT1*[29,30], *BAALC*[31], *EVI1*[32], *BRE*[33] of *VEGFC*[34] is associated with poor prognosis. Moreover, genome wide expression studies in both adult and pediatric AML have shown strong associations of established and less recurrent chromosomal aberrations with mRNA expression profiles[35] and revealed new disease entities, such as CEBPA mutated subgroup[36]. The identification of the genetic lesions and other molecular aberrancies that have biological and clinical implications is important since they allow classification, risk group stratification, targeted therapy prognostication and biological characterization of AML[9,11,28].

Treatment of pediatric AML and outcome

Treatment of AML is usually performed in two phases: A first induction phase aims to eradicate the bulk of tumor burden, which allows a complete remission of patients[37]. In most pediatric AML protocols, induction is based on intensive chemotherapy using a back-bone of cytarabine, an anthracycline such as daunorubicin and often etoposide[38]. Dosimetry of chemotherapeutics, disease management and supportive care have been optimized over past decades and in most high income countries, over 90% of patients achieve a complete remission[11,38,39]. Intensive additional courses of chemotherapy are applied in a second consolidation phase that is aimed at eradicating any residual disease and preventing relapse[37]. Well-defined patients may receive allogeneic stem cell transplantation (allo-SCT) from a matched sibling or unrelated donor, although it is generally not recommended for pediatric AML patients anymore[38]. In most protocols, patients are stratified in low, intermediate and high risk groups according to the cytogenetic characteristics of the leukemia and the initial response to therapy. Despite the high number of complete remissions, a significant portion (30-40%) of AML patients will suffer from a relapse. At relapse, salvage treatment is aimed at re-induction of a second complete remission (CR2), followed by consolidation with allogeneic stem cell transplantation. The protocols that have been applied by different national or collaborative study groups vary, but most regimens are based on cytotoxic chemotherapeutics similar to induction protocols, including primarily high dose cytarabine and an anthracycline[40].

Treatment failure

Chemotherapy has significantly improved prognosis for leukemia patients over the past decades[9,11,38,41], however improvements in long term overall survival rates seem to have reached a plateau level in children with AML at 60-70% (Figure 2)[38,39]. This is mainly due to a persistent portion of patients that suffers from relapse[11,39,42]. Relapsed AML patients respond poor to conventional salvage treatment and in a large portion of AML patients, current standard treatment options fail. Hence their outcome is poor, as two third of these patients die.

Different factors are known to contribute to treatment failure. First, pharmacokinetic factors are important in appropriate delivery of chemotherapeutic drugs. The standard chemotherapeutic drugs of most induction treatment protocols (cytarabine, daunorubicin or another anthracycline and etoposide) are administered in a body surface area adjusted manner to ensure optimal plasma concentrations and drug delivery to leukemic cells. However, upon administration, plasma concentration levels of the drugs are known to be highly variable[43,44]. Factors that are known to influence these plasma concentration levels are age, gender, base line WBC levels and kidney function. Improvements in pharmacokinetics can for example be achieved by alternative drug delivery methods: Liposomal delivery of DNR has resulted in significantly higher plasma levels and low conversion to metabolites[45]. Extra-medullary localization of AML e.g. in the central nervous system, skin, lymph nodes and soft tissues may occur in up to 40% of patients[46] and involve even more complex pharmacokinetics.
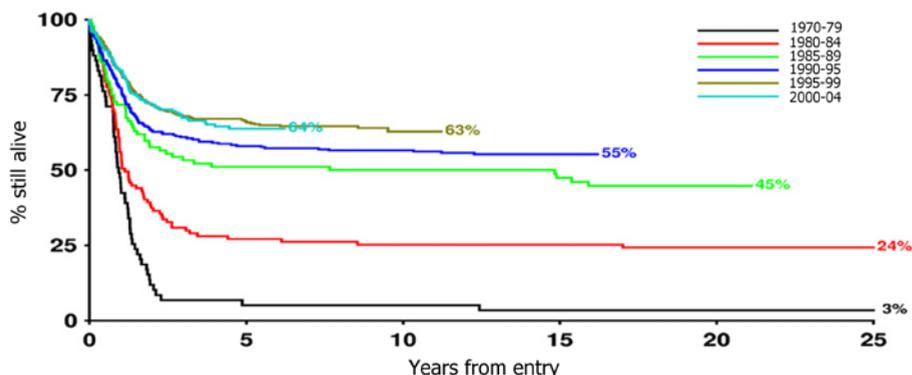
Figure 2. Overall survival of children (<15 years) with AML treated in MRC trials in the past three decades[39].

Even with optimal pharmacokinetics, the response to treatment may be poor due to intrinsic drug resistance of leukemic cells[47,48]. Known drug resistance mechanisms are for example over-expression of genes that encode drug efflux proteins such as the ATP-binding cassette (ABC) transporter gene *MDR1* or *MRP1* and *LRP* genes[47]. Drug uptake may also be impaired by an altered activity of nucleoside-specific membrane transport carriers, such as *hENT1*[49]. Alternatively, genes that impair apoptosis such as *BCL2* or *BCL-XL* are up regulated in AML during induction chemotherapy[50] and the expression of pro-apoptotic genes relative to anti-apoptotic proteins is associated with outcome[51] and predicts minimal residual disease[52]. Primary AML cells that survive *in vitro* Ara-C exposure show an increased expression of anti-apoptotic proteins and have an immature phenotype according to their high CD34 protein expression levels[53]. Such increased expression of anti-apoptotic proteins is also observed in CD34 expressing immature AML cells at diagnosis[54]. Especially immature AML cells are insensitive to chemotherapy.

Mechanisms that may allow even principally drug sensitive leukemic cells to escape damaging effects of chemotherapy are for example their oncogene induced capacity to proliferate which gives them a growth advantage over normal hematopoietic cells. AML cells interact intensively with their microenvironment[55] and these interactions are also frequently mentioned as factors that offer protection from chemotherapy[56]. Via for example integrin signaling or cytokines bone marrow stromal cells may confer leukemic cells growth signals[57] or inhibit apoptosis[58]. It has been shown that targeting bone marrow stromal cells overcomes such environment-mediated resistance *in vitro*[59].

Besides oncogenic aberrancies, polymorphisms in relevant genes that occur in human populations may be associated with drug resistance. Phamacogenomic studies aim to find the association of gene polymorphisms and drug resistance. This may involve polymorphisms in genes that are directly involved in drug metabolism, e.g. cytidine deaminase variants that are associated with Ara-C resistance[60]. Other examples of genetic polymorphisms that may confer drug resistance in AML patients are polymorphisms in the ABC transporter genes[61] or DNA damage repair genes[62]. Such polymorphisms may be associated with ethnical background, it has for example been shown that Hispanic ethnicity may be associated with reduced drug sensitivity of leukemic cells.

Targeted therapy in AML

Further intensification of chemotherapy in pediatric AML is impossible because of the severe side-effects of chemotherapy that would increase the toxicity related deaths (currently around 10%) and secondary cancers[38,63]. Moreover, the late-effects of

chemotherapy in childhood cancer survivors are becoming apparent, for example a reduced quality of life, impaired cognitive functions or infertility[64]. To overcome these problems, novel therapies are being developed that target AML according to its specific molecular aberrations. In contrast to conventional therapy, targeted therapies aim to be more leukemia specific, have a less broad mode of action and consequently are less toxic to healthy tissue[65]. Many targeted therapies aim to inhibit aberrant receptor tyrosine kinase (RTK) activation and put a hold on the aberrant proliferative signaling of type-I aberrations. RTKs play an important role in differentiation, proliferation and apoptosis of normal hematopoietic cells[66]. Most kinase mutations confer conformational changes to the protein, resulting in self-dimerization and consequently ligand independent activation. RTK inhibitors block this auto-activation, for example by restoring protein conformation, blocking kinase domain activity or preventing dimerization[67]. The kinase inhibitor Imatinib Mesylate (Gleevec®), which is directed against oncogenic BCR/ABL activity, has shown the power of this type of treatment in CML patients by improving response rates from 35% to over 90% when combined with chemotherapy, in comparison with conventional therapy alone[68]. The majority of chronic phase CML patients now achieve durable complete hematological remissions although the BCR/ABL transcript may still be detected in up to 10% of the patients[69,70]. Since the discovery of Imatinib, drug screenings have identified numerous kinase inhibitors, that target mutated kinases such as *FLT3*[71], *KIT*[72], *VEGFR*[73] or *PDGFR*[74]. Recent targeted therapeutics are designed to be more specific, as exemplified by the development of *FLT3* inhibitors: Most first generation RTK inhibitors, such as PKC412 show a broader kinase inhibiting activity, but seem to be active in *FLT3* mutated AML cells as they decrease auto-phosphorylation. Other drugs target RTKs more specifically, e.g. CEP-701 (Lestaurinib) blocks both wild type and mutant *FLT3*. More recently developed drugs, such as the *FLT3* inhibitor AC220 are designed to be even more specific[75]. RTK inhibitors that were tested as mono-therapies in clinical trials, predominantly in refractory or relapsed AML patients, showed little and temporary clinical efficacy[76]. Although moderate to strong reductions in peripheral or bone marrow blast counts were detected, CRs were never achieved. Results from a few randomized clinical trials in refractory and relapsed AML patients[76], but also a recent study in *de novo* AML in younger adults[77] show a possible benefit of RTK inhibitors in combination with standard chemotherapeutics.

Besides direct targeting of mutated kinases, the downstream signaling pathways and key mediating proteins are also subject of targeted therapy approaches. Aberrant RTK signaling, but also the presence of activating *NRAS* mutations in 5-20% of AML patients (depending on age) warrants interference in these pathways. *RAS* gene family members transduce signaling of RTKs to downstream pathways, such as the PI3-kinase pathway[78,79]. During protein synthesis, *RAS* proteins are subjected to post-translational modification processes that allow localization at the plasma membrane and exertion of their function in signal transduction. A crucial step in these processes is farnesylation of *RAS*, which can be inhibited by farnsyltransferase inhibitors (FTIs) that block the proper processing and localization of *RAS*[80]. However, clinical application of FTI mono-therapy in AML, e.g. with tipifarnib, has been disappointing with only low response rates (<30%) that were not associated with *RAS* mutations[81].

Inhibitors that target even further downstream of the RTKs are also being developed. Rapamycin for example is a drug that specifically targets mTOR, a molecule that mediates signal transduction within the PI3-kinase pathway. Rapamycin and derived drugs (rapalogs) show *in vitro* inhibitory effects on leukemic progenitors[82–84] and may sensitize leukemic cells to standard chemotherapeutics[85]. Phase I/II trials that follow this approach are currently being conducted[85,86].

Another strategy is to target AML according to epigenetic aberrations by application of DNA methyltransferase inhibitors[87] or histone deacetylase inhibitors[88]. A feasible

approach may also be to re-activate apoptotic pathways, e.g. by lowering the aberrant high expression of anti-apoptotic proteins such as *XIAP* with RNA silencing techniques[89] or by interfering with proteasome mediated protein degradation of pro-apoptotic proteins using proteasome inhibitors such as Bortezomib[90]. Immunotherapy, such as tumor cell-based vaccination strategies may provide elegant approaches, in which leukemia specific antigens are used to prime the adapted immune system of AML patients and allow a proper immune response to leukemic cells. Leukemia associated immunophenotypes may also be exploited in the development and application of antibody mediated therapies[91]. Gemtuzumab ozogamicin (GO; Mylotarg) for example, is an anti-CD33 anti-body that, conjugated with the drug calicheamicine, is internalized by leukemic cells and subsequently becomes cytotoxic. GO is effective as monotherapy in children with AML, as a response rate of 37% was achieved[92]. Improved CR rates and outcome may be achieved in combination with chemotherapy.

Relapse initiating cells

Despite optimization of therapy and all the new treatment strategies, relapse rates have not yet declined. This is disappointing, since the best opportunity to improve outcome for the AML patients is the prevention or optimal treatment of relapsed AML. In order to achieve this, it is important to characterize the few cells that are capable of initiating a relapse. These cells resist chemotherapeutic drugs, are capable of self-renewal and have the potential to repopulate a leukemia. Such characteristics are commonly present in normal immature (stem) hematopoietic cells.
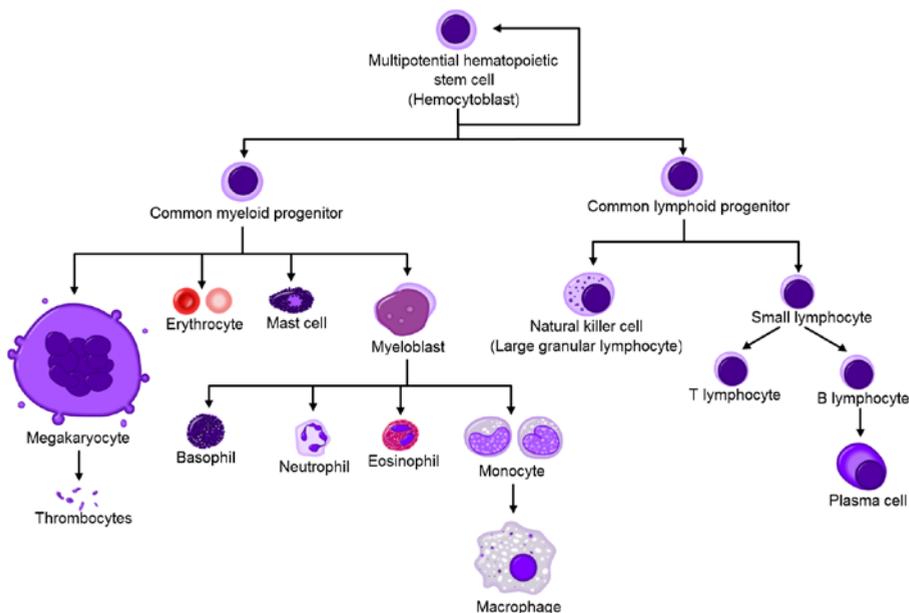

Figure 3. Schematic and simplified overview of normal hematopoiesis.

During hematopoiesis, blood cells are formed at an extremely high rate of up to 10 * 10^{10} new cells per hour[93]. Prior to birth, the primary organ of blood cell formation is the fetal liver and after birth blood cells are generated in the bone marrow. These organs contain a primitive type of blood cells, hematopoietic stem cells (HSC) that are multi-potent and thereby have the capacity to generate the cells of all different blood lineages

in a hierarchical manner. HSC replenish their own population by a process of self-renewal in which one daughter cell differentiates further and one daughter cell maintains a stem cell phenotype[94]. As HSC start to differentiate, progenitors arise that commit to the lymphoid or myeloid blood lineages (Figure 3). The progenitor cells differentiate and proliferate until the hematopoietic system is constituted of B-lymphocytes, T-lymphocytes and natural killer cells at the end of the lymphoid lineage. The myeloid cells that are formed are granulocytes, monocytes, macrophages, mast cells, erythrocytes and platelets. Different subtypes of the lymphoid and myeloid lineage cells can be distinguished on the basis of specific cell surface markers, which can be detected using flow cytometry analysis.
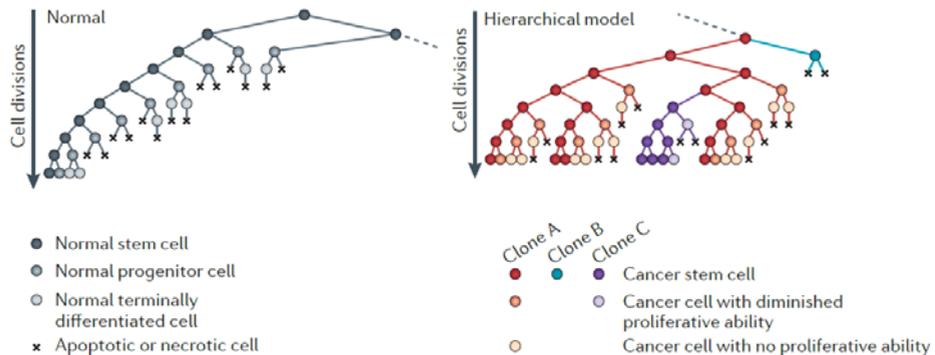

Figure 4. Schematic overview of the role of stem cells in normal hematopoiesis and leukemogenesis. Both of these processes involve a small, biologically distinct population of cells that have the capacity to self renew and to give rise to (more differentiated) progeny cells with a limited proliferative capacity. Accordingly, cancer stem cells retain at least part of the characteristics of their normal counterparts. (Adapted from Nguyen et al.[95])

It has been postulated that AML initiating cells are derived from the very early HSC (Figure 4). In particular when genetic lesions in essential regulatory genes accumulate, leukemic stem cells (LSC) develop that can initiate the leukemia. The bulk of leukemic blasts does not self-renew and shows limited proliferation[96]. When initial therapy is insufficient, patients that are in morphological (<5% blasts) or even complete remission (<5% blasts, regenerating bone marrow and no signs of leukemia elsewhere) may still harbor residual leukemic cells at the limits of detection. Molecular or flow cytometric detection of such minimal residual disease (MRD) in the bone marrow during follow-up of AML is highly predictive in both children and adults for the risk of relapse and subsequent outcome. This MRD contains the drug resistant cells that have the capacity to cause relapse. To accurately determine which patients have a high risk for relapse development, the physical identification of these relapse initiating cells (RIC) during disease progression is warranted. Flow cytometric detection of RIC exploits the aberrant expression of lineage and/ or maturation-associated cell surface proteins on leukemic stem cells that distinguishes them from normal immature myeloid cells or HSCs[97]. Application of this strategy allows for example accurate detection of leukemic stem cells within the normal HSC compartment as defined by a CD34+CD38- immunophenotype. Alternatively, functional stem cell characteristics may be used to identify the initiating fraction of leukemic blast cells (e.g. dye efflux[98,99] or aldehyde dehydrogenase[100–103]).

Although a subject of debate, the hierarchical stem cell model is substantiated by convincing evidence in AML[104]. The 'stemness' of a subpopulation of leukemic cells does not only imply for example their origin from normal counterparties or their *in* and *ex vivo* leukemia initiating capacity, but also an intrinsic drug resistance that is absent in more differentiated leukemic blasts[105].

These specific properties allow prospective identification and isolation of these leukemic stem cells (LSC) and thorough characterization, which may allow improvements in risk group stratification and the development of novel targeted therapy aimed to eradicate the LSC. This will ultimately result in circumventing relapse development and thereby improve overall survival of AML patients.

Outline of this thesis

CR rates in AML patients have improved dramatically over the past two decades, mainly due to intensification of treatment strategies and optimization of patient care. However, relapse rates have declined only marginally and are still approximately 35%. As a consequence, survival rates are reaching plateau levels with current treatment options. Targeted treatment options that aim to prevent or cure relapse may offer alternative approaches that will ultimately improve outcome. To develop new drugs and optimize such strategies, knowledge on the cells that drive disease progression and initiate relapse and to know their molecular and functional characteristics are crucial. The ultimate aim of the studies described in this thesis was to determine cellular and molecular factors that are involved in the relapse of AML.

First we studied relevance of several established molecular aberrations in disease progression, starting with the assessment of the stability of established type-I/II gene mutations (**Chapter 2**). Subsequently we assessed the frequency of established type-I/II gene mutations at first relapse and their prognostic relevance afterwards. (**Chapter 3**). Phenotypical and genotypical differences between diagnosis and relapse were previously described and confirmed by us and others. We aimed to assess the role of clonal selection in the phenomenon of mutational shifts between diagnosis and relapse (**Chapter 4**). To elucidate factors that may be involved in relapse, we performed gene expression profiling in relapsed AML patients. Differential gene expression profiles were analyzed between paired initial diagnosis and relapsed samples in order to unravel biological differences that may explain clinical differences between the two stages of the disease (**Chapter 5**). Relapse is caused by leukemic cells that survive initial therapy. We hypothesized that the characteristics of leukemic cells to survive therapy may already be apparent prior to treatment in part of the patients. These drug resistant characteristics may in part result from specific gene expression patterns. To assess drug resistance related gene expression in AML blasts, we correlated gene expression profiles of initial diagnosis samples with the *ex vivo* drug resistance of AML blasts from the same sample (**Chapter 6**).

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Chapter 1