

GENERAL INTRODUCTION

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1. AETIOLOGY OF ACUTE MYELOID LEUKEMIA

Erythrocytes, leukocytes and megakaryocytes, circulating in the peripheral blood are derived from the primitive pluripotent hematopoietic stem cell (HSC)¹. In adults the HSC mainly resides in the bone marrow, though it is proficient to circulate in the peripheral blood compartment. HSC have the ability of self-renewal and thereby maintain the pool of committed progenitors as well as to differentiate towards end stage cells. In healthy individuals only a small fraction of the HSC is dividing or differentiating. This process is tightly regulated via interactions with the bone marrow microenvironment, including hematopoietic growth factors and is responsive to functional demands.

Acute myeloid leukemia (AML) is a hematological disease represented by a wide variety of clinical and biological features. The incidence of AML is approximately 2–3 per 100.000 per year and increases progressively with age.

It is likely that most cases of leukemia arise through the acquisition of defects in the hematopoietic stem or restricted progenitor cells (Figure 1). Recent studies suggest, that occurrence of a mutation of the HSC is not strictly necessary: mutation of more committed progenitors may also be sufficient^{2–4}. These may in turn confer self-renewal properties to progenitors that are normally quiescent and lead to second mutations and a subsequent transformed phenotype. The degree of lineage commitment of the progenitor involved is thought to influence the leukemic characteristics.

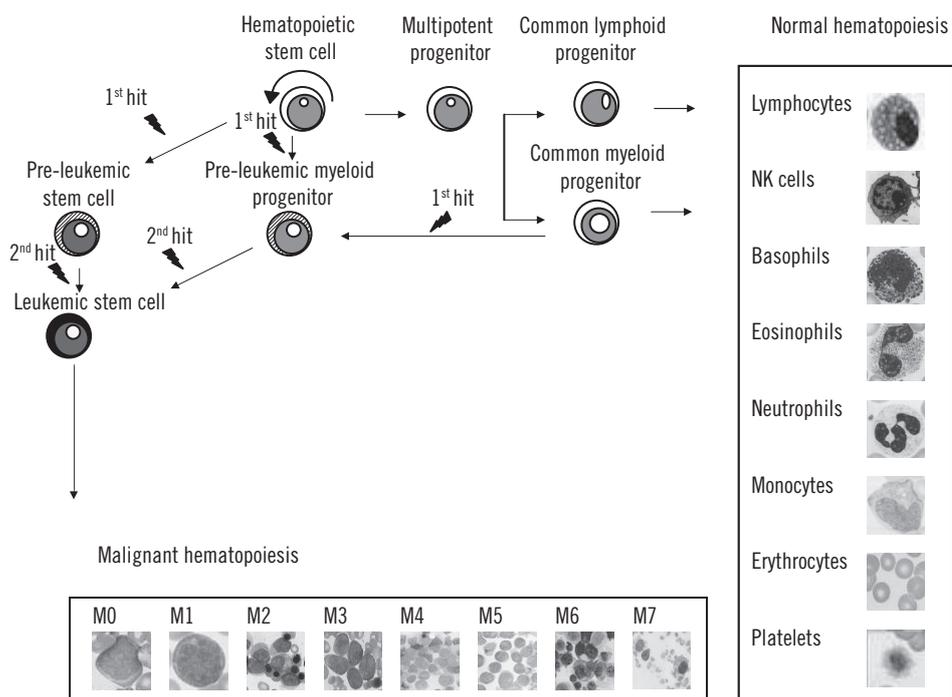


Figure 1. Schematic representation of the hierarchy of normal human hematopoiesis, resulting in formation of the different cell lineages. The figure also depicts a number of possible scenarios for leukemogenesis, via primary and secondary leukemic events in different blast populations defined by the FAB classification.

In the majority of cases, mutations are acquired during life. Sometimes this can be related to toxins like benzene^{5,6}, radiation^{7,8} or alkylating agents^{9,10}. Five to ten years after having received the latter therapy for previous malignancies, these patients can develop AML, usually preceded by a myelodysplastic phase and typically characterized by cytogenetic aberrancies in chromosome 5 or 7.

Secondary AML after the use of Topoisomerase-2 inhibitors, such as epipodophyltoxins¹¹ does not progress via MDS and arises after a shorter latency period of 1-3 years. The cytogenetic hallmark is a balanced translocation involving band 11q23 and a varying partner chromosome like t(6;11) or t(11;19). Secondary leukemia in general bears a poor prognosis.

Rare cases of familial AML have been described¹². Sporadic cases can arise due to mutations, which activate oncogenes or inactivate tumour suppressor genes, and disrupt the regulation of cell death, differentiation or division. Predisposing defects in DNA repair mechanisms like in Blooms syndrome¹³ and Fanconi Anemia (FA)^{14,15} accelerate the frequency at which new mutations are acquired.

1.2 Clinical presentation

AML is characterised by a differentiation arrest, which results in the accumulation of immature myeloid cells in the bone marrow and often in the peripheral blood as well. Blasts proliferate at the expense of normal blood cells. This results in anemia, leucopenia, lymphopenia and thrombocytopenia reflected by clinical symptoms as pallor, tiredness and infection as well as bleeding or bruising. Leucostasis may occur in patients with blast counts $>100 \times 10^9/L$. Hyper-leucocytosis is associated with an increased mortality early in the induction phase¹⁶.

In five percent of all patients leukemic cells emerge at extra-medullar localisations, e.g. the lymph nodes, liver or spleen; infiltration into the skin, soft tissues, testis, CNS or gingiva are observed as well. The balanced translocation 8;21 as well as CD56 (neural cell adhesion molecule)¹⁷ expression has been associated with a high incidence of extra medullar disease and its expression is associated with low CR rates and short OS¹⁸⁻²⁰.

1.3 Classification

Subtypes of AML have been classified on bases of morphological and cytochemical criteria as defined by the French American British (FAB) classification (Table 1)^{21,22}. Classically, cytochemical staining is applied to differentiate between AML and ALL. These stainings include Myeloperoxidase or Sudan Black which both are negative in ALL and positive in AML except for Mo/M7. Non-specific esterase staining is specific for monocytic leukemia. In addition, immunophenotyping is used to confirm and determine lineage commitment and is obliged for diagnosing AML-Mo and AML-M7.

In 2001 the World Health Organisation (WHO) reclassified both the hematopoietic and the lymphoid malignancies²³, based on morphologic, genetic, immunophenotypic, biologic and clinical features. With regard to the diagnosis of AML, the main conceptual change was the reduction in threshold of the percentage blasts in the bone marrow to distinguish AML from myelodysplastic syndrome (MDS): from 30% down to 20%. Moreover, patients with clonal recurring cytogenetic aberrancies are considered to have AML irrespective of the blast percentage. Four hierarchical new categories are defined in which leukemia is categorised on bases of disease aetiology (Table 1).

A specific subcategory of leukemia is represented by the biphenotypic leukemia. This subcategory probably arises from a multipotent progenitor cell and has a poor prognosis²⁴. The diagnosis of biphenotypic acute leukemia (BAL) is made according to EGIL (European Group of Immunological Classification of Leukemia's)²⁵.

Table 1. Classification of acute myeloid leukemia

World Health Organisation (WHO) ²³⁶	Incidence	FAB type ^{237,238}	Incidence
1 AML with recurrent genetic abnormalities	30-40%		
t(8;21)(q22;q22)		M2	25-30%
inv(16)(p13q22) or t(16;16)(p13;q22)		M4eo	5-15%
t(15;17)(q22;q12), (PML/RAR α) and variants		M3	5-10%
11q23 (MLL) abnormalities			
2 AML with multilineage dysplasia	10-15%		
Following MDS or MDS/MPD			
Without antecedent MDS or MPD, with dysplasia in at least 50% of cells in 2 or more myeloid lineages			
3 AML and MDS, therapy related	5-10%		
Alkylating agents/radiation-related			
Topoisomerase 2 inhibitor-related			
Others			
4 AML, not otherwise categorised	30-40%		
Minimally differentiated		M1	10-15%
Without maturation		M0	2-5%
With maturation			
Acute myelomonocytic leukemia		M4	5-15%
Acute monoblastic/monocytic leukemia		M5	15-25%
Acute erythroid leukemia		M6	-3%
Acute megakaryblastic leukemia		M7	5-10%
Acute basophilic leukemia			
Acute panmyelosis with myelofibrosis			
Myeloid sarcoma			

MDS indicates myelodysplastic syndrome; MPD myeloproliferative syndrome

On the bases of cytogenetic analysis two major groups of AML can be discriminated: one group with detectable chromosomal aberrations accounting for approximately 50% of all *de novo* AML and a second group without cytogenetically detectable karyotype abnormalities.

In the first group of patients cytogenetic analysis has resulted in the identification of clonal chromosomal rearrangements. Some of these rearrangements are molecular distinctive for certain FAB classes. For FAB M3 or acute promyelocytic leukemia (APL) this is t(15;17) (q22;q21), for M4eo inversion(16) (p13;q22) or t(16;16). Translocation t(8;21) (q22;q22) is associated primarily with M2, monocytic leukemia is frequently associated with 11q23. Part of these karyotypic abnormalities can be used to identify patient subgroups with specific clinical features or therapeutic responses as discussed in section 2.1.1.

1.4 Treatment

At present younger adults with acute AML are treated with remission induction regimens that include anthracyclins (daunorubicine or idarubicine) and cytarabine-arabino-side (ARA-C).

The aim of remission induction regimens is to clear the marrow from malignant cells and to allow repopulation of normal hematopoietic cells. The traditionally consensus definition of complete remission (CR) is defined as a bone marrow which appears normal morphologically and is functionally able to produce normal numbers of circulating cells with normal hematopoiesis of all cell types, i.e. less than 5% blast cells and a peripheral blood count of at least 1,500/ μL ($1.5 \times 10^9/\text{L}$) neutrophils and 100,000/ μL ($100 \times 10^9/\text{L}$) platelets.

The criteria of $<5\%$ blasts, is achieved in approximately 70–80% of the adults <60 years of age²⁶. Progress in the elderly patients has been much less conspicuous. Only approximately 40% to 60% of patients >60 years of age with AML achieve CR with ARA-C and an anthracycline such as daunorubicine or idarubicin or the anthracenedione mitoxantrone²⁷. However, only 20% to 30% of the younger patients (≤ 60 yrs) enjoy long-term disease survival, this percentage is even lower in the elderly patients (>60 yrs).

Various post-remission strategies have been explored to eliminate the residual leukemic cells further referred to as minimal residual disease (MRD), thereby preventing occurrence of relapse. For younger patients it has been demonstrated that remission duration can be prolonged by aggressive post-remission therapies that include allogeneic stem cell transplants, either from an HLA-identical sibling, or a matched unrelated donor or intensive chemotherapeutic regimens^{28,29,30}.

For allogeneic stem cell transplantation after achieving CR it is suggested that the merits of the transplant procedure outweigh the disadvantages with regard to probability of survival, although this strategy carries a high risk on short-term mortality and long-term morbidity as a result of graft versus host disease (GVHD). About 50% of the patients older than 60 years of age are not eligible for aggressive chemotherapy. Patients who are successfully treated with stem cell transplantation (SCT) may still experience relapse. Since the risk of mortality and morbidity of a second SCT or intensive chemotherapy is high, donor lymphocyte infusions (DLI)³¹ are occasionally applied. DLI encompasses transfer of white blood cells of the original SC donor into the recipient. This approach is based on the premise that the donors' lymphocytes will recognize the recipients' leukemic cells and eradicate them as a consequence of graft versus leukemia (GVL) effects.

In patients with relapsed or refractory disease re-induction regimens include high dose cytarabine as well as mitoxantrone, etoposide or fludarabine^{32,33}. Patients that achieve a second complete remission after relapse seem to benefit from allogeneic stem cell transplant³⁴. The duration of RFS after second remission is often reduced compared to the duration of RFS after first remission.

2. PROGNOSIS AT DIAGNOSIS

At diagnosis a number of factors can be identified that are predictive for patient responsiveness to induction chemotherapy as well as long-term survival. These factors can be subdivided into classical factors (2.1) and recently established parameters (2.2). In section 2.3 parameters currently examined for their prognostic impact are discussed.

2.1 Classical prognostic factors

2.1.1 Cytogenetics

The prognostic importance of cytogenetics has recently been updated by The Medical Research Council (MRC)³⁵ in the AML 10 trial, which included 1,612 children and adults up to 55 years of age. On the basis of response to induction treatment, relapse risk, and overall survival, three prognostic groups were identified.

The favourable prognostic group (25%) was defined by t(15;17), t(8;21) or inv 16; these are more frequently observed in the younger adult. Adverse cytogenetics (10%) included -7, -5, -5q, 11q23, complex karyotype and abnormalities regarding 3q; these are more common in the elderly adult AML patient. All other patients with either normal cytogenetics or rare cytogenetic aberrancies are considered to have an intermediate prognosis.

2.1.2 Age

The prognosis of AML becomes worse with increasing age. This is a result of changes in both the nature of the disease and patient characteristics³⁶.

In elderly patients disease is more likely to progress from preceding MDS, although in elderly patients data on the impact of AML preceded by MDS are conflicting³⁷. Moreover, elderly adult AML patients have a higher rate of unfavourable cytogenetics and clinical drug resistance is more common³⁸⁻⁴⁰. The adverse prognostic impact of higher age at diagnosis was however, shown to be independent of cytogenetic status^{41,42}.

A decrease in the performance status and an increase in co-morbidity in the elderly results in higher treatment morbidity and mortality and contributes to poor prognosis. As a result reduction of treatment intensity is required, but this in turn may lead to under-treatment of part the elderly patients which further contributes to poorer outcome when compared with younger patients⁴³.

2.1.3 White blood cell count

In a number of prospective studies a higher WBC count was observed to be a poor prognostic factor for achievement of CR as well as for survival duration in CR patients.

2.2 Recently established prognostic factors

2.2.1 Drug resistance

Apart from resistance mechanisms restricted to a particular drug, multi drug resistance (MDR) is an important factor that contributes to chemotherapy resistance in AML. MDR encompasses the ability of pathological cells to extrude functionally unrelated drugs that are supposed to eradicate such cells^{44,45}.

The ATP-binding cassette transporters (ABC) genes have been identified to code for transmembrane proteins that are able to transport a broad range of substances across cellular membranes, against a concentration gradient; a process that requires hydrolysis of ATP. At present 50 ABC transporters are known to be present in humans; these are sub-classified into seven families (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20 and White).

One of the main studied families is the MDR/TAP family that includes the full transporter P-glycoprotein (Pgp) that is encoded by the MDR1 gene (location 7q21.1). Over-expression of MDR1 is associated with multi drug resistance⁴⁶.

Substrates for which Pgp functions as efflux pump, include a number of anti-leukemic drugs like anthracyclins (daunorubicine and doxorubicine), anthracenes (mitoxantrone), vinca alkaloids (vinblastine and vincristine) and epipodophyllotoxins (etoposide).

Higher Pgp protein expression on leukemic blasts is found to be associated both with response to induction chemotherapy as well as with survival duration⁴⁷⁻⁵³. Some studies could not confirm these observations^{54,55}.

Other proteins involved in MDR are: multi drug resistance associated protein (MRP)^{56,57}, lung resistance related protein (LRP)⁵⁸, and breast cancer resistance protein (BCRP)⁵⁹.

Data on the prognostic impact of LRP revealed an association with leukemia free survival^{60,61} however, other studies could not confirm this finding⁶²⁻⁶⁴. For MRP data are conflicting with regard to treatment/survival prediction⁶⁵. For BCRP associations with disease outcome have not been reported. Substrates for MRP, LRP and BRCP are partly overlapping with those for Pgp however, differences exist.

The expression of multiple efflux pumps potentially worsens prognosis. Recently, patients expressing both MRP and LRP mRNA were observed to have poorer outcomes and worse 2-yr survival⁶⁶.

2.2.2 Apoptosis resistance

Apoptosis (from the Greek words *apo* = from and *ptosis* = falling) is used to indicate “dropping off” of petals or leaves from plants or trees. Nowadays the term apoptosis refers to one of the main types of programmed cell death (PCD). It involves a network of biochemical pathways that normally ensures a balance between cellular proliferation and turnover.

Failure of apoptosis creates a permissive environment for genetic instability and accumulation of genetic mutations. It promotes resistance to immune-based destruction and allows disobeying cell-cycle checkpoints when this would normally have led to apoptosis. Apoptosis resistance furthermore facilitates growth factor/hormone-independent cell survival, it supports anchorage-independent survival during metastasis, reduces dependence on oxygen and nutrients and confers resistance to cytotoxic anticancer drugs and radiation⁶⁷.

In the apoptosis process the cell becomes more rounded, due to the fact that the protein structures that are responsible for the cytoskeleton are digested by caspases. Secondly, the chromatin is degraded and condensed⁶⁸. The condensed chromatin then forms compact patches against the nuclear envelope in a process called pyknosis⁶⁹. The nuclear envelope becomes discontinuous and the DNA inside becomes fragmented (referred to as karyorrhexis). The nucleus breaks into several discrete chromatin bodies due to the degradation of DNA⁷⁰. Finally, plasma membrane blebbing occurs and the cell is phagocytosed, or breaks apart into several vesicles called apoptotic bodies, which are then phagocytised.

2.2.2.1 Apoptosis pathways

Apoptosis can be executed via two major pathways (Figure 2), the extrinsic pathway (receptor-mediated apoptotic pathway) and the intrinsic pathway (mitochondria-mediated apoptotic pathway), which are both well established and described in detail below. A third pathway, the Granzyme-B pathway is triggered by binding of cytotoxic T-lymphocytes (CTLs) to target cells and the release of Granzyme-B and perforin by the CTLs. Release of Granzyme-B can activate pro-caspase-3 and induce apoptosis. Serine protease inhibitors (Serpins) can interrupt this process.

Both extrinsic and intrinsic pathways have in common the activation of a group of cysteine proteases, called caspases, which carry out the cleaving of both structural and functional elements of the cell, resulting in characteristic morphological and biochemical changes.

The extrinsic apoptosis pathway is activated by binding of ligand of the TNF-super family to their receptors. Today this family has been identified to consist of 19 ligands that signal through 29 receptors.

The extrinsic apoptosis pathway is of particular importance for modulation of immune responses.

The family members that play a role in apoptosis consist of a death effector domain (DED) and a death receptor (DR). Upon binding of ligand to one of the DR (e.g. binding of CD95L/Fas-L to CD95/Fas) a protein or adaptor complex is formed, also referred to as DISC; death-inducing signalling complex.

FADD (Fas-associated death domain) is one of the proteins that form this complex.

This protein is able to activate caspase-8 via direct binding. Caspase-8 then activates the downstream caspases, which finally results in execution of apoptosis.

The intrinsic apoptosis pathway is controlled by proteins of the Bcl-2 family at the level of the mitochondria.

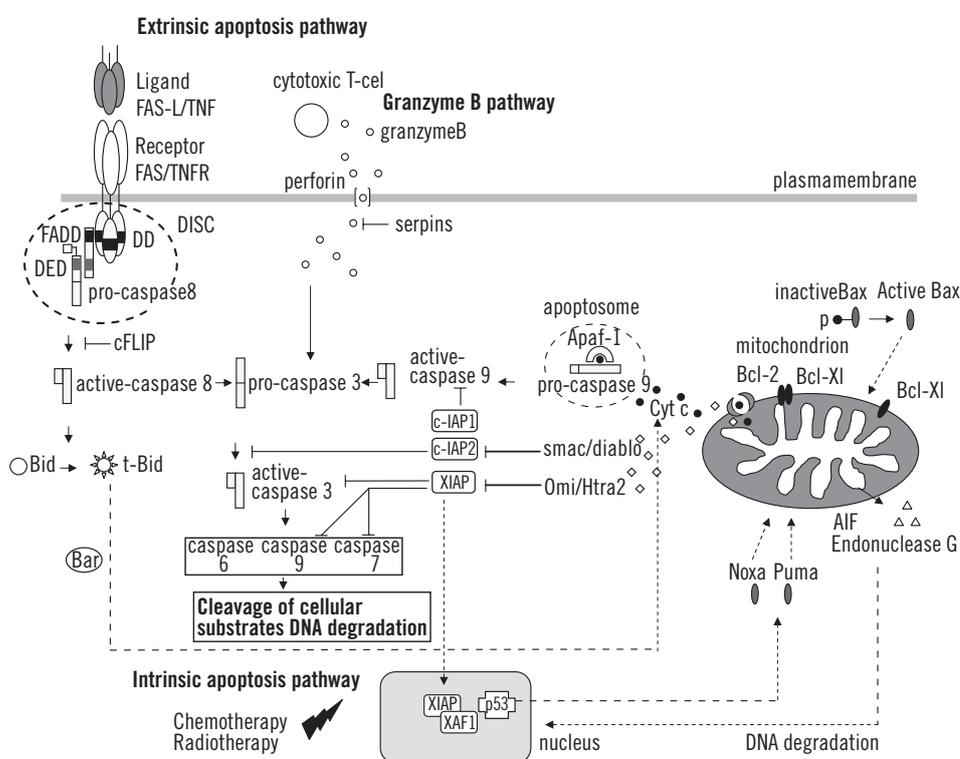


Figure 2. Simplified scheme depicting the extrinsic receptor mediated, the intrinsic or mitochondrial pathway and the Granzyme B mediated pathway of apoptosis induction.

The significance of the Bcl-2 family with respect to oncogenesis was first identified by the observation of the translocation $t(14;18)$, in approximately 70-80% of follicular lymphomas and in a lower proportion of the diffuse large B-cell lymphoma⁷¹⁻⁷³. This translocation results in the fusion of the Bcl-2 oncogene on chromosome 18 with IgH genes on chromosome 14, and leads to overexpression of Bcl-2 protein⁷⁴⁻⁷⁵.

It has become clear that Bcl-2 belongs to a larger family of proteins that are characterized by sequence homology domains, the so-called BH (Bcl-2 homology) domains.

Based on structure and function, the Bcl-2 family can be subdivided into three sub-

groups; anti-apoptotic Bcl-2 like proteins, pro-apoptotic Bcl-2 like proteins and pro-apoptotic BH₃-only proteins (Figure 3, left column).

The anti-apoptotic Bcl-2 like group contains proteins like Bcl-2, Bcl-XL, Mcl-1, Bcl-W and A1/Bfl-1; these proteins share 4 BH domains. Pro-apoptotic Bcl-2 like family proteins contain; Bax, Bak, Bok/Mtd and Bcl-Xs, which are characterised by BH1-3 domains. BH₃-only proteins possess only the BH₃ domain and are thought to bind their pro-survival counterparts Bad, Bik, Bim, Bid, Puma, Bmf, Harakiri and Noxa.

The Bcl-2 family of proteins consists of both antagonists and agonists that regulate apoptosis and compete through dimerization, thereby enhancing or antagonizing each other's function.

A number of proteins in the Bcl-2 family are pore-forming proteins that regulate ion and protein traffic over the mitochondrial membrane.

Individual Bcl-2 family members expression levels were shown to be independent prognostic indicators in a variety of malignancies including AML⁷⁶⁻⁸¹. High Bcl-2/Bax levels result in higher cell survival whereas low levels lead to apoptotic cell death⁸². Similar observations have been made for the Bcl-Xs/Bcl-XL ratio⁸³. Upon DNA damage or cellular stress, the mitochondria depolarise and Cytochrome-C is released. Cytochrome-C subsequently binds to the cytoplasmic protein apoptotic protease activating factor-1 (APAF-1) and pro-Caspase-9. Together they constitute the apoptosome; this complex subsequently activates specific caspases.

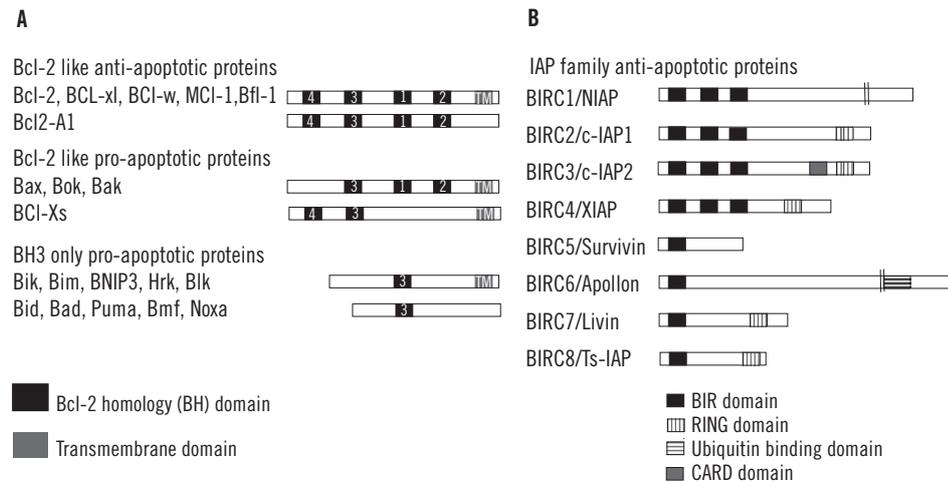


Figure 3. A. Overview of the different subclasses of Bcl-2 family proteins which share sequence homology in one or more Bcl-2 homology (BH) domains; depicted as black boxes. The transmembrane (TM) domains, depicted as grey boxes, localise the BCL-2 family members to the membranes of mitochondria and endoplasmatic reticulum or the nuclear envelope.

B. Schematic representation of human IAPs and their functional domains. BIR (Baculo viral IAP repeat domain), RING (ubiquitin ligase activity), UBD (Ubiquitin conjugation domain), CARD (Caspase recruitment domain).

2.2.2.2 Mediators of apoptosis

Caspases (cysteine aspartyl proteases) are the mediators of apoptosis. They are highly specific and synthesized as inactive precursors (pro-caspases). They can be subdivided into three categories on bases of their structure and function.

Caspases -2, -8, -9 and -10 are the initiator caspases. They have a long pro-domain that contains the regulatory sequences caspase recruitment domain (CARD) and the death effector domain (DED). These caspases are activated by binding to adaptor proteins such as APAF-1 and FADD and are the first to be activated upon commitment of the cell to die. Initiator caspases activate effector Caspases -3, -6 and -7.

These effector caspases have short pro-domains and no regulatory sequences and can cleave cellular substrates. Caspase-3 is the main effector caspase and is involved in the activation cascade of caspases responsible for apoptosis execution. At the onset of apoptosis it proteolytically cleaves poly (ADP-ribose) polymerase (PARP). It cleaves and activates sterol regulatory element binding proteins (SREBPs) between the basic helix-loop-helix leucine zipper domain and the membrane attachment domain. Further it leaves and activates Caspases -6, -7 and -9.

Caspases -1, -4, -5 and -11 to -14 are known as cytokine processors, involved in inflammation. These caspases do not fall within the scope of this thesis and are therefore not discussed in greater detail. An interactive effect of high levels of uncleaved Caspase-2 and -3 was observed, which denoted poor survival⁸⁴. The expression of caspases is mainly regulated by the Inhibitors of apoptosis proteins (IAP) family.

The IAPs are a family of anti-apoptotic proteins that are conserved across evolution. The common structural feature of all IAP family members is a ~70 amino acid zinc-binding fold termed the BIR domain, which is present in one to three copies (Figure 3, right column).

The human IAPs, XIAP, cIAP1, and cIAP2, have been reported to bind and potently inhibit Caspase-3, Caspase-7 and Caspase-9^{85,86}. Although quantitative studies are lacking, the IAP-family member Survivin also binds and inhibits some effector caspases. Moreover, at least some IAPs, such as XIAP, are capable of binding and suppressing specific initiator caspases such as Caspase-9. Smac/Diablo (Direct IAP Binding protein with low pI)^{87,88}.

XAF-1 and Omi/HtrA2 are proteins that can regulate IAP expression through binding IAPs by their IAP-binding motifs. IAP family members were shown to be widely and differentially expressed; moreover high expression levels were found to be associated with poor survival in AML^{89,90}.

The intrinsic and extrinsic apoptosis pathways are cross-linked by at least two proteins, to be known as Bar and Bid. Bar can interact with Caspase-8, Bcl-2 and Bcl-XL⁹¹. Bid is cleaved by active Caspase-8, while the truncated Bid (tBID) can induce Cytochrome-C release from the mitochondrium^{92,93}.

2.3 Factors under current investigation for their prognostic impact

2.3.1 Class I mutations

AML is a result of multiple “hits” or molecular changes that cause the leukemic phenotype. This leukemic phenotype results from the occurrence of both class I mutations; conferring a proliferative advantage to AML cells and class II mutations that lead to impaired hematopoietic differentiation. Collaborating events seem to be required to cause overt leukemia^{94,95}. Activating class I mutations include NPM1, FLT3, KIT, KRAS, NRAS and PTPN11.

2.3.1.1 NPM1

Initially, NPM1 (nucleophosmin also called B23 or numatrin), located on chromosome 5q35, was characterised as functioning in processing of ribosomal RNA⁹⁶, centrosome duplication⁹⁷ and regulation of p53⁹⁸ itself or p53 regulating factors like Rb⁹⁹, p19^{ARF100}, HDM2¹⁰¹.

The NPM1 gene was described to be over expressed in proliferating cells and down-regulated during apoptosis or differentiation. Mutations in NPM1 are amongst the most frequent (30-40%) acquired molecular abnormalities in acute myeloid leukemia (AML) and render a favourable prognosis in normal karyotype leukemia¹⁰².

NPM1 exon-12 gene mutations that include an insertion at residue 956 or deletion/insertion at residue 960 are the hallmark of a large AML subgroup with normal karyotype and frequently show myelomonocytic morphology.

NPM1 mutations frequently coincide with internal tandem duplications of the *fms*-like tyrosine kinase-3 gene (FLT3-ITD) and predict for better response to induction therapy and for favourable overall survival (OS) in the absence of FLT3-ITD¹⁰³.

The NPM1 protein product, shuttles between nucleus and cytoplasm¹⁰⁴. The concerted action of two alterations at mutant C-terminus, i.e. changes of tryptophan(s) 288 and 290 (or only 290) and creation of an additional nuclear export signal (NES) motif cause aberrant cytoplasmic localisation of the mutated NPM1^{105,106}.

Cytoplasmic NPM1 (NPM1c+) is associated with superior responsiveness to induction chemotherapy. Next to its potential role in regulating p53 function, NPM1c+ induces activation and translocation of the pro-apoptotic Bax protein to the mitochondria, thereby orchestrating mitochondrial dysfunction and apoptotic cell death¹⁰⁷.

2.3.1.2 FLT3

The *fms*-like tyrosine kinase-3 (FLT3) gene is located on 13q12¹⁰⁸ and is a member of the class 3 receptor tyrosin kinases (RTKs)¹⁰⁹. It encodes the FMS-like tyrosine kinase 3 (FLT3) receptor.

RTKs are constituted of 5 immunoglobine Ig-like domains, a juxta membrane (JM) domain, 2 intracellular tyrosine kinase (TK) domains and a C-terminal domain^{110,111}.

The FLT3 receptor is a membrane bound receptor for the hematopoietic growth factor FLT3 ligand (FLT3L or FL). Upon binding of FL, the FLT3-WT receptor dimerizes and becomes phosphorylated resulting in activation of pathways involved in proliferation and differentiation of cells, including phosphatidylinositol 3-kinase (PI3K)/Akt, Erk/MAPK and Janus kinase/signal transducers and activators of transcription (Jak/STAT) (Figure 4). Hence it was thought that alterations in signalling through FL expression or gain of function mutations might contribute to leukemogenesis.

Mutations of the FLT3 gene encompass in frame internal tandem duplications (ITD) in juxta membrane coding domain¹¹² (exons 14 and 15) as well as point mutations in the activating loop domain (exon 20).

The point mutations, occurring in about 7% of all AML, mostly affect asp835 in the second tyrosine kinase domain altering the conformation of the activation loop^{113,114}.

Less frequently FLT3 point mutations encompass ASP835Val, Asp835Asn, Asp835Ala, Asp835His, Asp835Glu.

All lead to constitutional activation of the receptor by autophosphorylation. ITD of the FLT3 gene (30-35% of all AML) occurs preferentially in normal karyotype; however incidence of 30% have been reported in patients diagnosed with APL¹¹⁵.

FLT3-ITDs are associated with poor overall survival and high relapse rate although not with impaired response to induction chemotherapy. FLT3-ITD signalling results in an anti-apoptotic cell status indirectly via the previously mentioned signal transduction

pathways involved in apoptosis regulation, culminating in phosphorylation of the pro-apoptotic protein Bad at Ser112 by Pim-1 kinase¹¹⁶.

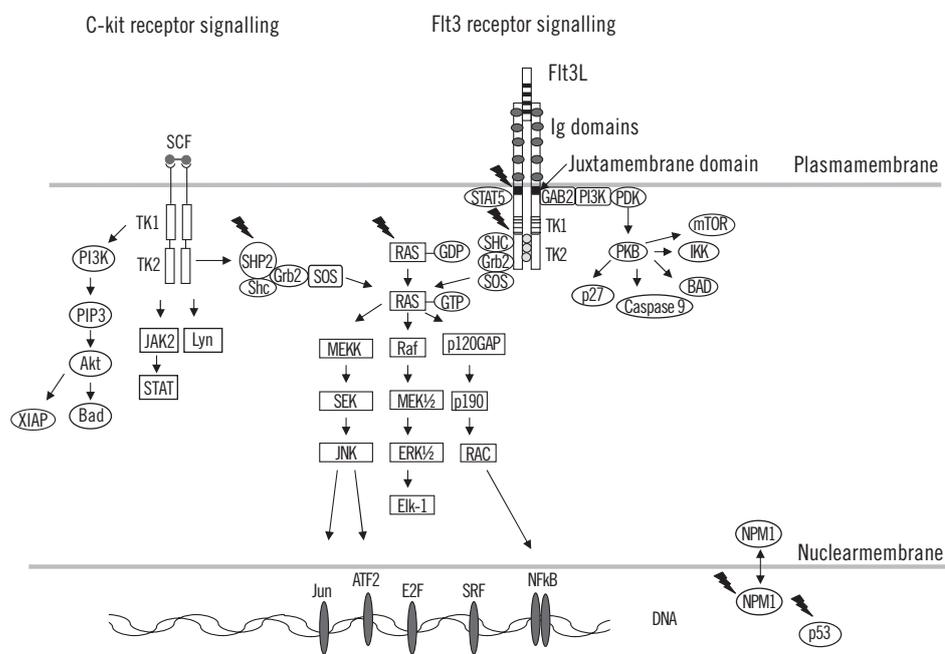


Figure 4. Downstream pathways of FLT3 and KIT signalling that directly or indirectly influencing apoptosis and/or proliferation.

2.3.1.3 KIT

The KIT proto-oncogene (4q11-q12) encodes a transmembrane receptor with a tyrosine kinase internal domain, which is expressed on the blast cell surface in most patients with AML or myelodysplastic syndrome (MDS).

Stem cell factor (SCF; also known as mast cell growth factor) is the ligand for this receptor. In concert the receptor and its ligand signal via pathways that stimulate the proliferation of different cell types including hematopoietic stem cells.

The receptor is expressed on more than 10% of blasts in 64% of the AML patients at diagnosis and 95% of relapsed AML patients. Oncogenic mutations in the KIT tyrosine kinase occur in 5-10% of the adult AML patients.

KIT mutations, mutKIT17 in particular, were recently shown to confer a higher risk of relapse, whereas both mutations in exons 17 and 8 adversely affected OS in AML with *inv(16)*¹¹⁷. The adverse impact of KIT mutations on relapse risk in *t(8;21)* AML may result from up regulation of the PI3K/Akt network¹¹⁸.

2.3.1.4 KRAS

KRAS (Kirsten rat sarcoma viral oncogene homolog; 12p12.1) encodes a protein that is a member of the small GTPase superfamily. Mutations include a substitution of one amino acid and are observed in a high number of malignancies.

KRAS mutations have an incidence of 5–10% in AML and are associated with *inv(16)* and FAB-class M4. NRAS (neuroblastoma RAS viral (VRAS) oncogene homolog; 1p13.2) is a member of the RAS family that has GTP/GDP binding abilities and GTPase activity. Their normal function may be as G-like regulatory proteins involved in the normal control of cell growth.

NRAS is activated in HL-60, a promyelocytic leukemia cell line. Oncogenic mutations are found around the hot spots at codons 12, 13, and 61 in 15–20% of the AML patients. Although associations with cytogenetic subgroups were described, no significant prognostic impact was found of mutated KRAS/NRAS for overall survival, event free survival, and disease free survival^{119,120}.

RAS mutations rarely co-exist with FLT3 aberrancies.

2.3.1.5 PTPN11

The PTPN11¹²¹ proto-oncogene (12q24) encodes for SHP-2, a widely expressed cytoplasmic protein tyrosine phosphatase¹²² functioning as a signalling transducer that is known to participate early in hematopoietic development.

Constitutional activating mutations are seen in Noonan's syndrome¹²³. Activating mutations in PTPN11 (mostly frequently occurring in exons 3, 4, 8 and 13), have been documented as a somatic event in a heterogeneous group of leukemia and rarely in solid tumours.

These mutations have been found to induce aberrant hyper activation of the RAS-ERK pathway, which in a mice models culminated in phosphorylation and destabilization of the pro-apoptotic protein Bim¹²⁴.

The PTPN11 mutation infrequently occurs in adult myeloid malignancies¹²⁵. The prognostic impact of PTPN11 mutations in adult AML has not yet been defined.

2.3.2 Class II mutations

Balanced chromosomal translocations most of the times result in chimerical in-frame proteins, with a transcription regulating function. CBF-AML, AML1-ETO and CBFβ-MYH11 gene fusions generated by *t(8;21)* or *inv(16)/t(16;16)* rearrangements represent these class II mutations.

Class II mutations link with higher age and are classically detected by cytogenetics.

The core binding factor (CBF) acute myeloid leukemia (AML) are defined by the presence of either *t(8;21)(q22;q22)* or *inv(16)(p13;q22)/t(16;16)(p13;q22)* chromosomal rearrangements that result in the disruption of CBFα (AML1) and CBFβ genes encoding CBF subunits, respectively.

Patients with CBF-AML account for 15% of the AML population. Their median age is significantly lower and their prognosis is better in terms of CR rate or time to relapse, than in patients with AML with normal karyotype or other chromosome aberrations. The activating class I mutations of KIT and RAS genes have a particular high incidence in CBF-AML.

The MLL (mixed lineage leukemia) gene is located in the 11q23 chromosomal band, consists of at least 37 exons and leads to a protein involved in regulation of transcription. A large number of abnormalities involving the MLL gene have been found in malignancies, including acute lymphoblastic leukemia (ALL), AML and myelodysplastic syndromes.

The MLL abnormalities can be divided into two categories.

The first category consists of MLL rearrangements, usually translocations or insertions, some of which are cryptic. In several cases, self-fusion of two parts of the MLL, lead to internal rearrangements called partial tandem duplication (PTD).

A second category of abnormalities is amplification of the 11q23 region, leading to the presence of multiple copies of the MLL gene. Numerical abnormalities of chromosome 11, such as trisomies or tetrasomies, also result in additional copies of the MLL gene.

Despite the variety of rearrangements involving the MLL gene, the overall prognosis of AML with this abnormality either balanced or unbalanced is unfavourable¹²⁶. Moreover, MLL rearrangements are found in 20% of the normal karyotype patients¹²⁷ and may thus help to differentiate AML with normal karyotype and poor prognosis from those with normal karyotype and a more favourable prognosis.

Acute promyelocytes leukemia (APL) AML-M₃ is an acute myeloid leukemia (AML) subtype characterized by proliferation of malignant promyelocytes with mature myeloid immune phenotype and the translocation t(15;17)(q22;q11), which results in the fusion of retinoic acid receptor- α (RAR α) gene on chromosome 17, which is involved in modulating myeloid differentiation, and the pro-myelocytic leukemia PML gene on chromosome 15.

The t(9;22)(q34;q11) resulting in the formation of the Philadelphia chromosome, is classically found in over 90% of adult patients with chronic myeloid leukemia (CML), however does also occur in 2% of AML patients. In AML it is associated with poor prognosis¹²⁸⁻¹³⁰.

2.3.3 Epigenetic aberrancies

According to the two hit model as proposed by Knudson in 1971, a phenotypic consequence of tumour suppressor genes (TSG) loss is not seen unless both alleles are affected, were in familial cancers one hit was inherited¹³¹.

Classically, oncogenesis or leukemogenesis is thought to originate from progressive accumulation of mutations in TSGs and oncogenes and/or chromosomal abnormalities. However, more recently it became apparent that epigenetic aberrancies also give a selective advantage to neoplastic cells¹³².

Epigenetic aberrancies are patterns of altered gene expression that are mediated by mechanisms that do not affect the primary DNA sequence and are heritable during cell division.

Early gene silencing events might addict cells to certain oncogenic pathways. Such epigenetic addiction could predispose cells to accumulate mutations which stimulates further oncogenic/leukemogenic progression¹³³.

The main epigenetic aberrations include gain or loss of DNA methylation and altered patterns of histon acetylation.

DNA methylation involves addition of a methyl group to the cytosine pyrimidine ring. Approximately 60-70% of all cytosine bases coupled to guanine bases by phosphodiester linkage (CpGs) are methylated. Unmethylated CpGs are grouped in clusters called CpG islands that are present in the 5' regulatory regions of many genes.

In tumorigenesis, gene promoter CpG islands can acquire abnormal hypermethylation, which then results in transcriptional silencing^{134,135} or become hypomethylated resulting in increased transcription^{136,137}.

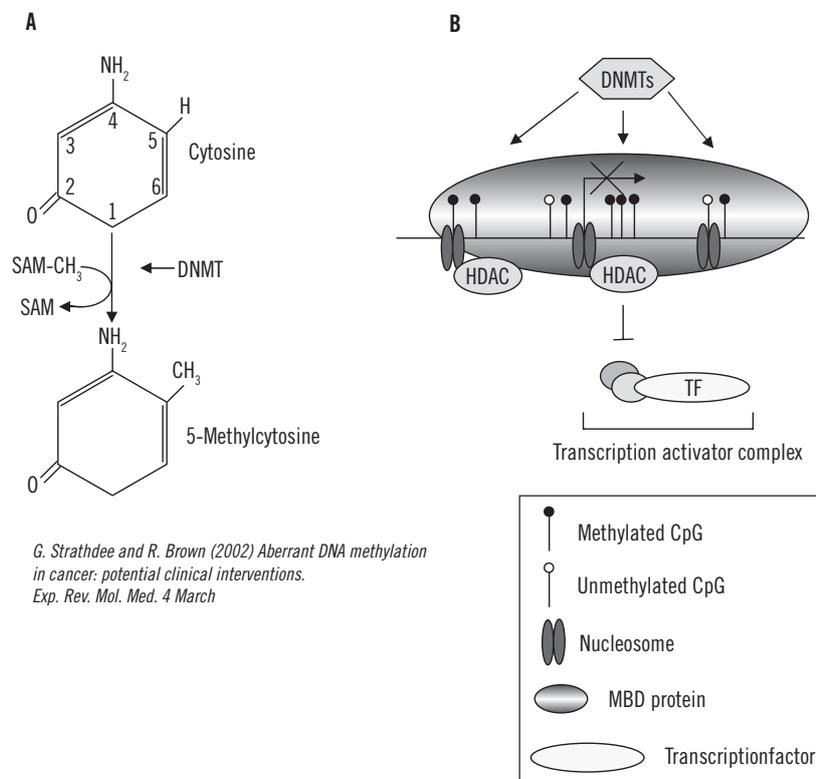
The inverse relation between hypermethylation and repressed transcription however only accounts for the promotor regions and not for the transcribed parts of genes¹³⁸.

DNA methylation may affect the transcription of genes in two ways. First, the methylation of DNA may itself physically impede the binding of transcriptional proteins to the gene but secondly and likely more important, methylated DNA may be bound by proteins known as Methyl-CpG-binding domain proteins (MBDs).

MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodelling proteins that can modify histones, thereby forming inactive silent chromatin (Figure 5).

The previously discussed protein nucleophosmin (NPM1) was recently discovered as a human histone chaperone that becomes acetylated, resulting in the enhancement of chromatin transcription¹³⁹.

CpG island methylation in translational medicine at present comprises its use as marker for cancer cells, as marker for tumour behaviour and as target for therapy.



G. Strathdee and R. Brown (2002) *Aberrant DNA methylation in cancer: potential clinical interventions*. *Exp. Rev. Mol. Med.* 4 March

Figure 5. A. DNA methylation occurs at cytosine-phosphate-guanine (CpG) sites and results in the conversion of the cytosine to 5-methylcytosine. This reaction is catalyzed by the DNA methyl transferase (DNMT) enzyme.

B. CpG sites are found in a high density near gene promoters; CpG islands. Methylation of these CpG islands as orchestrated by many accompanying proteins may influence gene transcription.

2.3.4 Gene expression profiles

Of the classical prognostic indicators as mentioned in section 2.1, cytogenetics is the most powerful predictor for patient survival.

However, 35–50% of the patients belong to the heterogeneous intermediate cytogenetic risk group. These include patients with a normal karyotype, rare chromosomal aberrations or yet unidentified aberrations. Molecular markers like mutations in the FLT3, NPM1 as well as the MLL gene have enabled us to sub-stratify this patient group with respect to survival probabilities and define therapeutic targets.

High expression of individual genes have shown to carry independent prognostic impact in predicting survival especially in the normal cytogenetic risk group, for example the BAALC gene (Brain and Leukemia Cytoplasmatic gene)^{140,141}.

Signatures that appear to separate cytogenetically normal AML patients into prognostic subgroups have been described^{142,143}. The use of gene-expression profiling by microarray studies has improved the molecular classification of adult AML.

Mutations in CEBPA occur in approximately 7 percent of patients with AML, most with a normal karyotype, and predict a favourable outcome¹⁴⁴.

High levels of expression of EVI1, which occur in approximately 10 percent of cases of AML, predict a poor outcome. The clinical implications of all these prognostic genetic findings in normal karyotype AML have recently been discussed¹⁴⁵.

Although subgroup stratifications seem to improve, gene-expression signature-based classifiers predicting outcome for individual patients with greater accuracy are needed.

Next to sub stratification of the cytogenetic intermediate risk group, progress is being made as to the identification of other pathogenetically relevant candidate genes, both for the identification of disease markers, diagnostic classification and discovery of therapeutic targets.

By means of gene expression profiling the downstream effects of specific known or yet to be identified gene perturbations can be determined. Genes that are involved in treatment resistance pathways like MDR and apoptosis are of special interest in this respect.

3. PROGNOSTIC FACTORS AFTER INDUCTION TREATMENT

3.1 Complete remission

Classically achievement of complete remission (CR), as well as the number of cycles of chemotherapy necessary to reach CR, was considered as the main prognostic factors after induction treatment.

3.2 Presence of minimal residual disease

Recently the level of minimal residual disease (MRD) was established as prognostic factor highly predictive for patient survival^{146–149}.

The term MRD refers to the low amount of cell present in remission bone marrow after chemotherapeutic treatment in absence of clinical symptoms.

The retrospectively determined prognostic value of MRD cell frequencies will currently be applied in prospective randomised phase-3 studies e.g. HOVON 42a (described in greater detail at www.hovon.nl).

Several techniques are being used or have been adapted for monitoring absolute MRD cell frequencies during the course of the disease.

Classically karyotyping and Fluorescent In Situ Hybridisation (FISH) are applied to detect cytogenetic abnormalities, which can be found in 30–40% of all AML patients. The use of both methods is limited since their sensitivity is relatively low 10^{-2} and either metaphases (cytogenetics), or meta- or interphase cells are obliged (FISH).

Quantification of MRD by means of amplification of specific DNA sequences is only possible if these sequences are tumour specific.

Potential targets for such approach are fusion genes like PML-RAR α , AML1-ETO and inv16, which are available in 30% of AML patients in Western Europe.

Junctional regions of rearranged Immunoglobulins (Ig) and TcR¹⁵⁰ can be used for this approach in a minority of patients (10%). These rearrangements are considered to be patient specific, since they are specific for each lymphocyte and thereby for each clonal leukemic malignancy.

Mutations can also serve as target for monitoring at DNA level; like NPM1 and FLT3-ITD¹⁵¹.

Since the NPM1 mutation is a frequent abnormality in AML patients without known genetic marker; the mutation may represent a new target to monitor minimal residual disease in AML and a potential candidate for alternative and targeted treatments.

Recently, a study was performed using RQ-PCR assays for NPM1 mutations to monitor and quantify MRD¹⁵². The utility of NPM1 mutation for sequential monitoring was confirmed in a small patient cohort by the observation that the mutation remains stable between diagnosis and relapse¹⁵³.

In case of the FLT3-ITD, detection can also be performed on mRNA-derived cDNA. Products of Reverse Transcriptase (RT)-PCR are used in case of aberrant expressed genes like WT-1^{154,155} (80-100% of the adult AML patients) or preferentially expressed antigen of melanoma (PRAME; 30% of the adult AML patients)¹⁵⁶.

Transcripts of fusion genes like PML-RAR α ^{157,158} or AML1-ETO are also used for MRD detection in AML. In general QT-PCR approaches reach a sensitivity of 10^{-4} to 10^{-6} . Cross-contamination by non-patient specific transcripts as well as relative instability of RNA is an important pitfall of this approach.

Immunophenotypical detection^{159,160} is based on the presence of leukemia associated immunophenotypes (LAPs), which are unusual or aberrant immunophenotypes that differentiate malignant leukemic cells from normal myeloid bone marrow cells.

These LAPs concern, cross-lineage antigen expression (e.g. expression of lymphoid markers on myeloid cells), asynchronous antigen expression (e.g. co-expression of early markers with those associated with advanced myeloid maturation), overexpression of antigens (e.g. abnormal high levels of myeloid or primitive markers) and/or ectopic expression (e.g. presence of antigens that are normally not expressed on hematopoietic cells).

This method is easy to perform and sensitive, detecting 1 malignant cell in 1,000 to 10,000 normal cells, but needs substantial knowledge of normal bone marrow differentiation patterns.

LAPs can be defined in 80-100% of all AML patients. Low but variable staining of normal cells as well as the occurrence of immunophenotypic shifts offers a limitation of this approach.

4. CHARACTERISTICS OF MINIMAL RESIDUAL DISEASE

Outgrowth of residual cell results in relapse of disease and is fatal in almost all cases. Several leukemia intrinsic and extrinsic mechanisms that can exert influences that contribute to emergence and outgrowth of MRD are discussed in this section.

In theory the immune system continuously recognizes tumour antigens and eliminates malignant cells (immune surveillance).

Especially when the leukemic burden is minimal for example in CR, leukemic cells may have developed strategies to circumvent immune recognition. These strategies include, secretion of immunosuppressive factors that block the synthesis of Th1 type cytokines in T cells¹⁶¹. Other mechanisms of importance are development of T-cell tolerance¹⁶², resistance to cytotoxic T lymphocyte pathways¹⁶³ and lack of expression of “co-stimulatory” molecules required for activating the immune response¹⁶⁴. Ineffective presentation of tumour antigen antigens in the MHC class-2 molecules as a result of the presence of class-2 associated invariant peptide (CLIP)¹⁶⁵ may also contribute to high MRD frequencies.

Next to defects in immune surveillance, the intrinsic mechanisms that leukemic cells employ to survive chemotherapy may explain emergence of MRD. By unravelling such mechanisms, selective therapeutic approaches can be developed both at diagnosis and under MRD conditions.

Two main cellular drug resistance characteristics resulting in survival of malignant cells are Multi Drug Resistance and Apoptosis Resistance.

One would expect these MRD cells to be characterised by a more resistant cellular phenotype compared to diagnosis. This would either result from selection of subpopulations present at diagnosis with higher drug efflux ability of or a more apoptosis resistant phenotype, or from up-regulation of drug efflux pump activity or anti-apoptotic protein function.

However, studies in which these cellular resistance profiles determined in MRD cells were compared to profiles of whole blast population and subpopulations at diagnosis revealed somewhat puzzling results.

With regard to Multi Drug Resistance, during follow-up no significant differences as compared to diagnosis were observed in Pgp, MRP and BCRP function¹⁶⁶. While MRD cells of patients obtained in remission even displayed a more apoptosis sensitive protein profile compared to diagnosis¹⁶⁷. These findings appear contradictory. For MDR we showed that the function of the Pgp and MRP drug efflux has a Gaussian distribution over the whole blast cell population, with no particular preference for sub-compartments like the stem cell compartment (unpublished data). We thus hypothesized that therapy results in survival of cells that are not different in resistance characteristics from the whole population.

This is supported by the finding that Pgp mediated efflux is not significantly different between the compartment with the presumed highest cell survival (i.e. stem cells) and the bulk of the blasts (unpublished observations). Resulting in percentage cell survival, that is mainly dependent on the resistance parameter in the whole cell population i.e. the larger the efflux the higher the percentage cell survival and distribution is remained during the course of the disease.

For apoptosis resistance characteristics a similar normal distribution may be present at diagnosis, but apoptosis related protein expression likely is dependent on external factors that depend on the number of blasts present.

For adequate eradication of malignant cells a proper bio-distribution of chemotherapeutic drugs is a prerequisite.

Both intrinsic cellular drug resistance as well as patient specific pharmaco-kinetics can result in inadequate levels of chemotherapeutic drugs and may thereby explain high frequencies of residual cells after chemotherapy.

This advocates that inadequate pharmacokinetics which includes absorption and disposition (distribution, metabolism and excretion) of drugs may result in increased chemo survival of both malignant as well as normal cells, not only by direct effects but also indirect via reduced damage to the bone marrow stromal cells.

These stromal cells normally protect against apoptosis by producing soluble factors (cytokines and chemokines; IL-1, IL-6, SDF-1, SCF, thrombopoietin), via direct cellular contact as well as via interactions with bone marrow matrix (fibronectin, proteoglycans). The discovery of the role of multiple hematopoietic growth factors and their receptors in the orchestration of stem cell self-renewal and differentiation has led to the perception that deregulation or autonomous activation of these critical regulators of hematopoiesis may play a role after induction chemotherapy in outgrowth of MRD. Stromal derived factor 1 (SDF-1) produced by the bone marrow microenvironment can directly interact with the chemokine receptor 4 (CXCR-4/fusin/LESTR) present on the hematopoietic stem cell.

SDF1 is suggested to play a role in stem cell trafficking, as is shown by reduced bone marrow hematopoiesis in SDF-1-deficient mice and the chemotactic effect of SDF-1 on CD34⁺ progenitor cells. CXCR4 expression on CD34⁺ is associated with reduced OS¹⁶⁸. Stroma was suggested to create a permissive environment by enhancing adhesion of AML blasts to fibronectin¹⁶⁹ and thereby protecting blasts from apoptosis¹⁷⁰ or via direct up regulation of anti-apoptotic proteins¹⁷¹.

This permissive environment allows the AML blast to further accumulate aberrancies. CXCR-4 expression was observed to be higher in FLT3-ITD patients compared to FLT3-WT patients¹⁷².

5. TARGETED TREATMENT STRATEGIES

Although improvement in CR rates and long term survival have been made by combining different type of drugs and performing dose escalation studies, the basic therapeutic approach of chemotherapeutic treatment regimens have changed little in the past decade.

Better understandings of the molecular abnormalities that occur in the leukemic cell have led to the identification of several new potential targets.

5.1 Targeting the apoptosis pathway

Defects in the apoptosis pathway contribute to resistance to a variety of chemotherapeutic drugs¹⁷³.

Bcl-2 is a potent inhibitor of caspase-dependent as well as caspase-independent apoptosis¹⁷⁴. Down-regulation of anti-apoptotic proteins like Bcl-2 may restore blast susceptibility towards chemotherapy induced apoptosis.

G3139 (Genasense, Genta, Berkeley Heights, NJ) an 18-mer phosphorothioate Bcl-2 anti-sense binds human BCL-2 mRNA. Pre-clinical studies revealed that when administered alone or in combination with chemotherapy G3139 down regulates BCL-2 expression *in vivo* and *in vitro*, resulting in apoptosis¹⁷⁵⁻¹⁷⁸. G3139 has been tested in refractory AML and ALL: in 9 out of 20 patients CR was achieved whereas 3 patients showed response without hematological recovery¹⁷⁹.

A phase 3 clinical trial by the CALGB is now investigating the addition of BCL-2 antisense to both induction and post-remission therapy.

Inhibitors of apoptosis proteins (IAPs) suppress cell death by inhibiting both upstream and downstream caspases¹⁸⁰⁻¹⁸².

XIAP is the most widely expressed IAP family member¹⁸³ and was shown to be the most potent inhibitor of caspases¹⁸⁴⁻¹⁸⁶. High XIAP expression levels are associated with reduced survival^{190,187}, whereas down-regulation of XIAP by anti-sense oligonucleotides induced caspase activation and sensitised HL-60 cells to Ara-C induced cell death¹⁸⁸. Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad anti-tumour activity and clinical studies will soon start.

The ubiquitine pathway is essential for degradation of intracellular proteins. Proteasome inhibitors can influence the relative levels of oncogenic proteins. NFkappa B¹⁸⁹, c-Myc¹⁹⁰, c-Fos¹⁹¹, c-Jun¹⁹² and some of the apoptosis proteins like the XIAP¹⁹³ are important targets for proteosomal degradation.

Bortezomib (PS341), a newly approved drug for multiple myeloma, is an inhibitor of the proteasome. It has demonstrated *in vitro* synergistic activity with histone deacetylase inhibitors (HDAC-inhibitors) as well as potential activity as single agent in leukemia^{194,195}. Bortezomib in addition to standard chemotherapy have been shown to be feasible in AML in phase II studies.

5.2 Targeting signal transduction

Intracellular interactions mediated by cytokines, growth factors influence gene transcription via intercellular signalling pathways. Constitutive activation of kinases as well as dysfunction of transcription factors and adaptor proteins have been linked to leukemogenesis. Translocations have been described to constitutively activate tyrosine kinases.

Tyrosine kinase inhibitors that target the constitutive FLT3 signal, thereby inducing apoptosis have been applied in clinical phase I and phase II trials in relapsing or refractory AML and MDS patients. Examples are CEP-701 (Cephalon, Frazer, PA)^{196,197}, PKC-412 (Novartis, Basel, Switzerland)¹⁹⁸, SU11248¹⁹⁹ and SU5416²⁰⁰⁻²⁰², both from (SuGen, New York, NY).

The tyrosine kinase inhibitor Imatinib Mesylate (STI-571, Novartis, Basel, Switzerland) was designed for BCR/ABL tyrosine kinase inhibition, but it was shown to inhibit c-ABL, KIT, and platelet-derived growth factor-receptor (PDGF-R) as well.

Response to treatment in KIT positive AML patients with STI-571 is reported to have efficacy in some studies²⁰³, while others did not find *in vivo* activity^{204,205}.

It has recently been reported that PKC-412, is also a potent inhibitor of mutant KIT proteins that are resistant for Imatinib Mesylate (KIT D816V and KIT D816Y)²⁰⁶.

The C-terminal prenylation of Ras, necessary for association with cell membrane and transforming activity is mediated by farnesyltransferases.

A large number of farnesyl transferase inhibitors (FTIs), that interfere with Ras signalling by precluding farnesylation of Ras and thereby its transfer to the plasma membrane²⁰⁷, have been developed.

In phase II clinical trials R115777 (Tipifarnib, Zarnestra, Johnson & Johnson, New Brunswick, NJ) was shown to be well tolerated in older adults with poor-risk AML, and to have activity in refractory AML²⁰⁸ while being modestly active in newly diagnosed AML patients²⁰⁹.

Several other FTIs are under clinical investigation like SCH66336 (Lonafarnib, Sarasar, Schering-Plough, Kenilworth, NJ)²¹⁰⁻²¹² and L778123 (Merck & Co, Whitehouse Station, NJ)²¹³.

Recently it was demonstrated that the PI₃-kinase-Akt-mTOR (mammalian target of rapamycin) pathway is constitutively activated in about 60% of AML patients cells.

In vitro, low doses of the specific inhibitor of mTOR, rapamycin (Calbiochem-Novabiochem, La Jolla, CA) and its analogues such as CCI-779, RAD001 and AP23573, block the phosphorylation of the classical targets of this kinase and inhibit the proliferation of leukemic progenitors without affecting the growth of normal hematopoietic progenitors²¹⁴.

5.3 Targeting epigenetic aberrancies

Epigenetic modifications that lead to gene transcriptional repression have been associated with malignant transformation and are new targets in the treatment of AML. In contrast to genetic deletions that cause irreversible loss of gene function, epigenetic gene silencing mediated by DNA methylation and histone deacetylation can be reversed via pharmacologic inhibition of DNA methyltransferases and histone deacetylases, respectively.

Normal patterns of gene expression, hematopoietic differentiation and apoptosis may be restored and disease response obtained.

Decitabine, 2'-deoxy-5-azacytidine, can inhibit DNA methyltransferases and reverse epigenetic silencing of aberrantly methylated genes²¹⁵.

For decitabine anti-leukemic effects have been postulated for quite some time²¹⁶.

More recently, dose-dependent hypomethylation after decitabine at low doses was shown in patients with myeloid leukemia and correlated with response.

Increasing the decitabine dose was not accompanied by further hypomethylation²¹⁷.

This observation was substantiated in phase I study on relapsed/refractory leukemia, decitabine inhibited DNA methyltransferases and appeared to induce the most responses (11 of 17 or 65%) at lower dose (15 mg/m² for 10 days). Fewer responses were seen when the dose was escalated or prolonged (2 of 19 or 11%)²¹⁸.

HDACs and HATs are enzymes which catalyse deacetylation and acetylation of histones. Their dynamic balance provides accurate regulation for gene transcription and gene expression.

Disbalance of these enzymes can bring disorder to proliferation and differentiation in normal cells, and lead to the initiation of tumour.

Although only 20% of the whole genome is controlled by HDACs, key processes for survival, such as proliferation, and differentiation have been strictly linked to HDAC enzyme functioning. The use of HDAC inhibitors (HDACi) has been proposed for the treatment of neoplastic diseases.

Their effectiveness has been suggested for a number of liquid and solid tumours, particularly acute promyelocytic leukemia (APL).

A phase I/2 study on the combination of 5-aza-2'-deoxycytidine (azacytidine) and the histone deacetylase inhibitor valproic acid (VPA) in patients with advanced leukemia, including older untreated patients showed that the combination of epigenetic therapy in leukemia was safe, active and associated with transient reversal of aberrant epigenetic marks²¹⁹.

5.4 Targeting aberrancies in the Fanconi Anemia pathway

The 13 FA-genes identified to date are thought to be essential for specific DNA repair processes.

Cells which are defective for one of the FA-genes display genomic instability and are hypersensitive to DNA cross-linking agents^{220,221}.

This cellular phenotype is associated with increased cancer risk as observed in patients with fanconi anemia (FA).

These patients are characterized by a diversity of clinical symptoms including an increased risk to develop malignancies in particular myelodysplastic syndrome and acute myeloid leukemia (AML), but also solid tumours.

In the past years a number of papers have reported on the potential role of acquired disturbances in the FA-pathway in sporadic cancers^{222,223}.

Hypermethylation of the FANCF promoter has been described to occur in various tumour types such as those of the ovaries²²⁴, cervix²²⁵, lungs and oral cavity²²⁶.

Also in AML the occurrence of inherited and somatic abnormalities in the FA-genes has been studied²²⁷⁻²²⁹.

Although epigenetic events in the FA-pathway are infrequently found in AML, they may play a role in generating genetic instability in a subset of sporadic AML patients. The use of DNA cross-linkers in AML patients with aberrancies in the FA-pathway for clinical use as alternative treatment remains to be investigated.

5.5 Other potential therapeutic targets

Antigens displayed by the leukemic cells are prominent targets to eradicate malignant blasts. CD33 is myeloid specific antigen and is expressed on the surface of >90% of all leukemic blasts.

Naked antibody (HuM195) against CD33 internalises after binding to the antigen. This offers opportunities for conjugates with toxins and radioisotopes. The concomitant toxicity probably results both from eradication of CD33 positive progenitor cells and CD33 positive stem cells.

Gemtuzumab Ozogamicin (GO) antibody conjugated to calicheamicin and has demonstrated efficacy in clinical trials^{230,231}.

Novel AML antibody targets like CD123 (IL-3R alpha chain), CD44^{232,233} and CLL-1 (C-type lectin like molecule 1)²³⁴ have recently been identified to target blasts and AML stem cells.

Whether these are selective for AML stem cells and thereby constitute promising targets remains to be elucidated.

Immunologic approaches are applicable if tumour associated antigens are identified.

In the case of WT1 peptide vaccination, the WT1 gene product serves as target antigen. WT1 anti-sense inhibits proliferation of CD34⁺ cells and high expression of WT1 is associated with poor prognosis.

AML specific fusion genes (PML-RAR α and AML1-ETO) have also been suggested as candidate targets.

PR1 a peptide derived from the primary granule proteins of granulocytes and their precursors has also suggested as vaccination target²³⁵.

6. SCOPE OF THE THESIS

Intensification of chemotherapeutic regimens and the introduction of stem cell transplantations has resulted in improved survival prognosis of acute myeloid leukemia in the past decades.

Unfortunately, this progress has been made at the expense of considerable treatment related mortality and morbidity. This urged to develop risk adapted treatment strategies.

The ever-increasing number of such alternative targeted treatment regimens demands new prognostic factors to identify the patients that may best benefit from new treatment strategies.

Classical risk stratifying factors such as cytogenetics and age at diagnosis are insufficient for this purpose. In the past decade a high number of genetic and physiological factors have been discovered to carry prognostic impact with respect to patient survival.

One of the physiological factors and hallmarks of leukemogenesis that exert such impact on survival is apoptosis resistance. For example, apoptosis related protein and single or combined gene expression at diagnosis was previously shown to strongly predict survival.

In *chapter 2* the applicability of the MLPA (Multiplex Ligation-dependent Probe Amplification) technique, originally developed for DNA, for simultaneous monitoring of the expression of a large number of apoptosis related genes (RT-MLPA) is evaluated.

This chapter furthermore describes the feasibility of RT-MLPA for the characterisation of small cell subpopulations, like MRD cells.

Using this multi-gene expression approach we argued that the prognostic value of apoptosis related gene expression at diagnosis would be strengthened compared to the parameters used thus far. *Chapter 3* describes the construction and validation of such outcome predicting apoptosis related gene expression profile.

Changes in apoptosis related protein expression of the blasts have previously been observed to occur during the course of the disease and thereby determine the adequacy of further treatment. Extended characterisation of surviving cell populations after induction is therefore warranted.

Since MRD cells have very low frequencies the RT-MLPA method described in *chapter 2* was applied to describe the characteristics of MRD cells in terms of apoptosis resistance in *chapter 4* by multi-gene expression of blasts obtained at diagnosis with those of MRD cells in corresponding samples.

Ongoing clinical research in AML focuses on drugs that target unique genomic aberrations, protein structure/functional alterations or deregulated cellular pathways that are specific for molecular subgroups of patients rather than embracing cytarabine-based chemotherapy as a standard approach for all patients.

Newly identified characteristics of leukemic cells, like the presence of FLT3-ITD, NPM1 mutations as well as aberrant gene methylation all eventuate in a more apoptosis resistant phenotype and constitute novel therapeutic targets for specific patient subgroups.

We have evaluated one of these in terms of effects on the frequencies of MRD cells after chemotherapy.

Chapter 5 evaluates the possibility to use MRD frequencies as potential short term read out for efficacy of therapy targeting FLT3 gene mutations. *Chapters 6* and *chapter 7* assess methylation aberrancies of tumour suppressor genes and DNA repair associated Fanconi Anemia genes, as potential therapeutic targets for either demethylating or DNA cross-linking agents.

Finally, the findings described in this thesis and the proposed perspectives that may guide subgroup directed therapeutic approaches are discussed in the chapter summary and conclusions.

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