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RAS, *KIT*, *FLT3* and *JAK2* Gene Mutations in Acute Myeloid Leukemia (AML) with inv(16) and t(8;21): Incidence and Relevance on Clinical Outcome

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Amtierender Dekan:

1. Berichterstatter:

2. Berichterstatter:

Tag der Promotion:

Content

Abbreviations

ACN	Acetonitrile
Allo-SCT	Allogeneic stem-cell transplantation
AML	Acute myeloid leukemia
AMLSG	AML Study Group
APL	Acute promyelocytic leukemia
ATRA	all-*trans*retinoic acid
Auto-SCT	Autologous stem-cell transplantation
BM	Bone marrow
bp	Base pair
CBF	Core-binding factor
CBF*B*	Core-binding factor β
CBFs	Core-binding factors
CEP	Anti-cancer compound
CI	Confidence interval
CIR	Cumulative incidence of relapse
CALGB	Cancer and Leukemia associated Group B
CMPD	Chronic myeloproliferative diseases
CNS	Central nervous system
CR	Complete remission
CRi	Complete remission with incomplete hematological recovery
CSR	Cycle sequencing reaction
DFS	Disease-free survival
DHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ED	Early death
EDTA	Ethylene diaminetetraacetic acid
EFS	Event-free survival
EML	Extramedullary involvement
ETO	Eight twenty-one
EUN	N-ethyl-N-nitorsourea
FAB	French-American-British

FISH	Fluorescence *in situ* hybridization
FTIs	Farnesyl transferase inhibitors
GISTs	Gastrointestinal stromal tumors
HAM	High-dose cytarabine and mitoxantrone
HiDAC	High-dose cytarabine
HOVON	Hemato-Oncologie voor Volwassenen Nederland
HPV	Human papilloma virus
HR	Hazard ratio
HSC	Hematopoietic stem cell
HSCs	Hematopoietic stem cells
HSPCs	Hematopoietic stem/progenitor cells
HUGO	Human Genome Organization
IC	Idarubicin, cytarabine
ICE	Idarubicin, cytarabine, etoposide
IDAC	Intermediate-dose cytatabine
IE	Idarubicin, etoposide
ins/del	Insertion/deletion mutations
inv(16)	Inversion inv(16)
ITD	Internal tandem duplication
JAK	Janus kinase
JM	Juxtamembrane
KI	Kinase insert
LDH	Lactate dehydrogense
LSCs	Leukemia stem cells
MCD	Mast cell disease
MDS	Myelodysplastic syndrome
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MPD	Myeloproliferative disorders
MRC AML 10	Medical Research Council's 10th AML trial
MYH11	Myosin heavy chain 11
NCAM	Neural cell adhesion molecule
OR	Odds ratio
OS	Overall survival

PB	Peripheral Blood
PCR	Polymerase chain reaction
PCR-RFLP	PCR with restriction fragment length polymorphism
PDGFRa	Platelet-derived growth factor- receptor-α
PDGFRB	Platelet-derived growth factor- receptor-β
PKC	Protein Kinase C
PML	Promyelocytic leukaemia
RARA	Retinoid-acid receptor-α
RD	Resistant disease
RFS	Relapse-free survival
rpm	Rotation per Minute
RT-PCR	Reverse transcription-polymerase chain reaction
RTKs	Receptor tyrosine kinases
RUNX1	Runt-related transcription factor 1
S-AML	Secondary AML
SCF	Stem cell factor
SCT	Stem cell transplantation
SMMHC	Smooth-muscle myosin heavy chain
SNL	Sinonasal lymphomas
STAT	Signal transducer and activator of transcription
STI571	Signal transduction inhibitor number 571
t(8;21)	Translocation t(8;21)
t-AML	Therapy-related AML
TE	Tris-EDTA
TEAA	Triethylammonium acetate
TK	Tyrosine kinase
TKD	Tyrosine kinase domain
TKIs	Tyrosine kinase inhibitors
TM	Transmembrane domain
Tris	Tris (hydroxymethyl) aminomethane
UV light	Ultraviolet light
WBC	White blood cell
WHO	World Health Organization

1. Introduction

1.1. Acute myeloid leukemia

Acute myeloid leukemia (AML) is a heterogeneous malignant hematopoietic disorder characterized by clonal expansion of immature myeloid cells in the bone marrow, blood or other organs. The affected cells underly an uncontrolled proliferation and impaired differentiation program. Typically, the cells are blocked at various maturation steps and are resistant to cell death (Martelli et al. 2006). AML accounts for approximately 80% of all adult leukemias. Complete remission (CR) can be achieved in approximately 60% to 70% of adult AML following appropriate induction therapy and more than 15% of the AML patients (about 25% of those who attain CR) can be expected to survive 3 or more years and may be cured (Tallman et al. 2005). Increased morbidity and mortality during induction therapy appear to be directly related to age, as well as other adverse prognostic factors including central nervous system (CNS) involvement, severe infection at diagnosis, elevated white blood cell (WBC) count, treatment-induced AML, or history of myelodysplastic syndrome (MDS) (Campos et al. 1992; Lowenberg et al. 1999).

Standard in the diagnosis of AML is the cytologic examination of Wright-Giemsa or May-Grünwald Giemsa stained blood and bone marrow smears by light microscopy allowing reproducible classification by experienced morphologists in most of the cases. Until 1999, the most widely used AML classification was the French-American-British (FAB) classification that described the degree of differentiation and the lineage of leukemia based on predominantly morphological criteria (Table 1) (Bennett et al. 1985). The FAB classification also included cytochemical stains. With the intention to link previous, predominantly morphologic classification systems with newly emerging scientific data, the current World Health Organization (WHO) classification of hematopoietic neoplasms was recently implemented. This new classification comprises four major categories of AML (Table 2) (Harris et al. 1999).

Table 1. FAB classification of AML (Bennett et al. 1985)

Category	Morphology	Incidence (%)
M0	AML with differentiation	3
M1	AML without maturation	15-20
M2	AML with maturation	25-30
M3	Acute promyelocytic leukemia (APL)	5-10
M4	Acute myelomonocytic leukemia	25-30
M5	Acute monocytic/monoblastic leukemia	2-10
M6	Acute erythroleukemia	3-5
M7	Acute megakaryoblastic leukemia	3-12

FAB: French-American-British; AML: Acute myeloid leukemia.

Table 2. WHO classification of AML (Harris et al. 1999)

Category	Morphology	Incidence (%)
AML with recurrent cytogenetic translocations	AML with t(8;21)(q22;q22), *AML1*/*ETO*	5-12
	AML with (15;17)(q22;q11-12), (*PML*/*RAR* α) and variants,	10-15
	AML inv(16)(p13q22) or t(16;16)(p13;q11), (*CBFb*/*MYH11*)	5 3-5
	AML with 11q23 (*MLL*) abnormalities	
AML with multilineage dysplasia	With prior myelodysplastic syndrome	10-15
	Without prior myelodysplastic syndrome	
AML and mylodysplastic syndromes (MDS), therapy-related	Alkylating agent–related	5-10
	Epipodophyllotoxin-related (some maybe lymphoid)	
	Other types	
AML not otherwise categorized	AML minimally differentiated	40-50
	AML without maturation	
	AML with maturation	
	Acute myelomonocytic leukemia	
	Acute monocytic leukemia	
	Acute erythroid leukemia	
	Acute megakaryocytic leukemia	
	Acute basophilic leukemia	
	Acute panmyelosis with myelofibrosis	

WHO: World Health Organization; AML: Acute myeloid leukemia; *ETO*: Eight twenty-one; *CBFb* : Core-binding factor β; *MYH11*: Myosin heavy chain 11.

1.2. Prognostic subgroups based on cytogenetic findings

In the nineties a number of prospective and retrospective studies have clearly demonstrated, that cytogenetic aberrations detectable in the leukemic cells at the time of diagnosis are the most important prognostic factors for clinical outcome (Grimwade et al. 1998). Based on these findings AML is now categorized in three major cytogenetic groups: low-risk group, intermediate-risk group and high-risk group (Table 3). Using conventional cytogenteic analysis acquired clonal chromosome aberrations (ie, reciprocal translocations, inversions, insertions, deletions, trisomies, and monosomies) can be detected in the pretreatment marrow of 50% to 60% of adults with de novo AML (Figure 1).

Table 3. Risk group assignments of AML with frequent cytogenetic findings (Grimwade et al. 1998)

Favorable-Risk Group	
Balanced structural rearrangements	t(15;17)(q22;q12-21)
	t(8;21)(q22;q22)
	inv(16)(p13q22)/t(16;16)(p13;q22)
Intermediate-Risk Group	
	Normal karyotype
Balanced structural rearrangement	t(9;11)(p22;q23)
Unbalanced structural rearrangements	del(7q)
	del(9q)
Numerical aberrations	–Y
	+8
	+21
	+22
Unfavorable-Risk Group	
	Complex karyotype
Balanced structural rearrangements	inv(3)(q21q26)/t(3;3)(q21;q26)
Unbalanced structural rearrangement	del(5q)
Numerical aberrations	–5
	–7

AML: Acute myeloid leukemia; del: deletion; inv: inversiton; t: translocation.

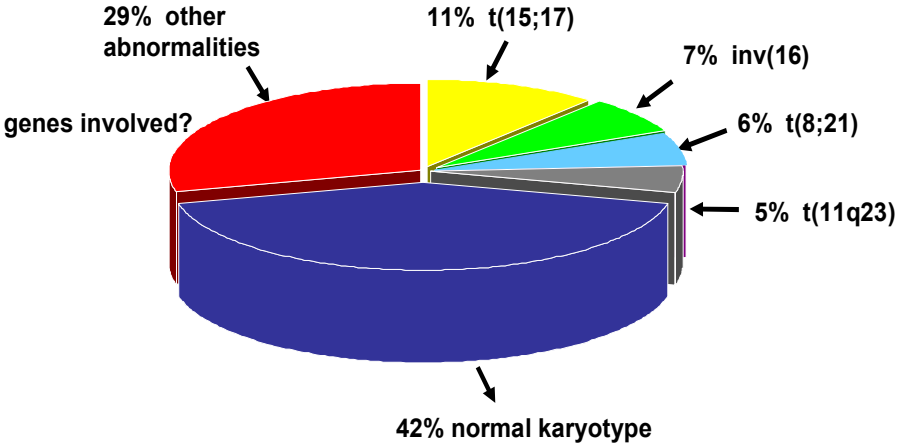

Figure 1. Genotypic diversity of AML based on the different cytogenetic subgroups. AML: acute myeloid leukemia.

Based on the proposed WHO classification, the first group is defined by leukemias exhibiting specific gene fusions resulting from balanced translocations or inversions like the translocation t(15;17) associated with acute promyelocytic leukemia (APL), the inversion/translocation inv(16) and the translocation t(8;21). AML associated with either t(8;21) or inv/t(16) represents the so called core binding factor (CBF) leukemias, affecting the same transcription factor complex. In addition, chromosomal translocations of band 11q23 involving the *MLL* gene belongs to this cytogenetic subgroup of AML. These four groups are now recognized as specific genetically defined subsets within the WHO classification.

The second group is defined by the large subset of AMLs with normal karyotype which comprises approximately 45% to 50% of AMLs. In these cases submicroscopic lesions can not be detected by conventional chromosome banding analysis.

The remaining 30% of AMLs exhibit other abnormalities, mainly aneuploidies, such as trisomies, monosomies or deletions, including the cases with complex karyotype. Interestingly, to date almost no gene has been identified that is targeted by these abnormalities.

In recent years a large number of gene aberrations have been identified in normal karyotype AML, some of them providing novel insights into the pathology mechanisms of leukemia. In addition, some of these gene alterations are of

prognostic relevance and are now being used for risk-stratification of the patients.

1.2.1. Core binding factor (CBF) acute myeloid leukemia

Inversion or translocation of chromosome 16 [inv(16)(p13q22) or t(16;16)(p13;q22)], hereafter abbreviated inv(16) and translocation t(8;21)(q22;q22) abbreviated t(8;21) belong to the most common cytogenetic abnormalities in AML (Erichkson et al. 1992; Liu et al. 1993). At the molecular level, these genomic aberrations are characterized by the disruption of genes encoding different subunits of the core binding factor family. Patients with CBF AML are considered to have a favorable outcome, in particular when treated with cytarabine-based consolidation regimens. However, a significant proportion of these patients relapse and survival after 5 years is about 50%. These findings suggest that additional genetic lesions are underlying the clinical heterogeneity.

1.2.1.1. Molecular biology of CBF AML

The core-binding factors (CBFs) are heterodimeric transcription factors that serve, in part, as overseers and regulators of hematopoietic ontogeny (Speck et al. 2002). The CBF family consists of three distinct DNA binding *CBFα* subunits (*RUNX1*, *RUNX2* and *RUNX3*), and a common non-DNA-binding *CBFβ* subunit that is encoded by *CBFB*. *RUNX1* — the first mammalian CBF gene to be isolated — has been known by a number of names, originally *AML1* — which remains the most commonly used name in the literature — but also *PEBPA2B* and *CBFA2*, in 1999, which was renamed by the Human Genome Organization (HUGO), and is now formally known as runt-related transcription factor 1 — RUNX1. The *CBFα* subunit binds directly to the enhancer core DNA sequence on target genes, whereas the beta subunit does not bind the DNA directly but increases the affinity and stabilizes the binding of the alpha subunit to the DNA. The *RUNX1* and *CBFB* genes are required for hematopoietic stem cell (HSC) emergence during development, and are key regulators of hematopoiesis at several steps (Speck et al. 2002). The *CBFβ* subunit is encoded by *CBFB* on chromosome 16q22 and is disrupted by inv(16)(p13q22) or t(16;16)(p13;q22) (Liu et al. 1993). *RUNX1*, located on chromosome band 21q22, is disrupted by t(8;21)(q22;q22) (van der Reijden et al. 1997).

1.2.1.1.1. inv(16)(p13q22) and t(16;16)(p13;q22)

At the molecular level, inv(16)(p13q22) and t(16;16)(p13;q22) both result in the fusion of the *CBFβ* gene from chromosome 16q22 with the *MYH11* (myosin heavy chain 11) gene from chromosome 16p13 (Liu et al. 1993; Lowenberg et al. 1999). The *MYH11* gene encodes a smooth-muscle myosin heavy chain (*SMMHC*) (van der Reijden et al. 1993). Two novel fusion genes, *CBFβ/MYH11* and *MYH11/ CBFβ*, are created as a result of the inversion of chromosome 16 but only the first appears to be critical in leukemogenesis. Several studies have demonstrated that the genomic breakpoints within *CBFβ* and *MYH11* are extremely variable, and at least eight types of *CBFβ/MYH11* fusion transcripts have been identified by reverse transcription-polymerase chain reaction (RT-PCR), which the most common is referred to as type A detected in approximately 85% of patients with AML and inv(16) (Claxton et al. 1994; Liu et al. 1995).

The mechanism whereby the *CBFβ/MYH11* fusion gene leads to malignant transformation remains to be fully elucidated (Liu et al. 1995). Data from murine models appear to support the hypothesis that *CBFβ/MYH11* interferes with normal hematopoiesis in a dominant-negative manner. Mice lacking the *CBFβ* gene or which are heterozygous for a *CBFβ/MYH11* allele produce an identical phenotype in which the animals lack fetal liver hematopoiesis, resulting in early embryonic death (Castilla et al. 1996; Wang et al.1996).

1.2.1.1.2. t(8;21)(q22;q22)

The translocation t(8;21)(q22;q22) is a balanced translocation between chromosomes 8 and 21, resulting in the fusion of the acute myeloid leukemia 1 (*AML1*) gene from chromosome 21q22 with the eight twenty-one (*ETO*) gene on chromosome 8q22 (Erickson et al. 1992; Nucifora et al. 1993). In this translocation, the first five exons of *AML1* are fused to almost the entire *ETO* gene (Erickson et al. 1992; Miyoshi et al.1991). The *AML1* N-terminus contains the DNA binding motif; the C-terminus of the *ETO* gene contains a domain that appears necessary for transcriptional activation (Ito Y. 1996). The *ETO* gene encodes a protein with two putative zinc-fingers and several proline-rich regions. It is presumed to function as a transcription factor (Miyoshi et al. 1993).

In t(8;21)(q22;q22) AML, the *AML1/ETO* fusion gene is created on the derivative chromosome 8. This gene encodes the *AML1/ETO* fusion transcript that is believed to play a primary role in leukemogenesis (Erickson et al. 1992; Nucifora et al. 1993). Loss of either *AML1* or *ETO* results in embryonic lethality with complete lack of definitive hematopoietic stem cells (HSCs) (Wang et al. 1996). In addition, it was recently reported that *AML1*+/– adult mice suffer from a 50% reduction of long-term repopulating stem cells (Sun et al. 2004). Although it is yet not fully understood how the *AML1-ETO* fusion gene contributes to leukemogenesis, it is thought that one key mechanism is the suppression of *AML1*- and *C/EBPα*- dependent activation of genes responsible for myeloid development (Licht et al. 2001; Pabst et al. 2001). In vivo and ex vivo analyses demonstrated that alterations in the differentiation pattern and proliferative capacity of murine hematopoietic cells expressing the fusion gene (Rhoades et al. 2000; Fenske et al. 2004). However, numerous murine in vivo models documented that *AML1-ETO* on its own is not able to induce leukemia (Castilla et al. 1996; Rhoades et al. 2000), suggesting that introduction of additional genetic changes might contribute to t(8;21)-positive AML pathogenesis through their constitutive activation.

Since the results of these studies appear very similar to those obtained from studies of mice lacking the *CBFβ* gene or which are heterozygous for a *CBFβ/MYH11* allele, it is reasonable to postulate the existence of a common pathway that leads to the leukemic phenotype in CBF AML. The leukemogenic role of the *AML1-ETO* protein is further supported by preliminary evidence showing that *AML1-ETO* chimeric mice created with the "knocking-in" methodology may develop myelomonocytic leukemia when a second "hit" event is induced with a chemical mutagen at a sublethal dose (Schwieger et al. 2002).

1.2.1.2. Clinical features and prognostic factors in CBF AML

1.2.1.2.1. inv(16)/t(16;16)-positive AML

AML with inv(16) is found in approximately 10% to 12% of all AML cases, predominantly in FAB subtype M4eo that is characterized by an abnormal eosinophilic differentiation (Le Beau et al. 1983). Inv(16) has been found to be associated with higher WBC count, percentages of peripheral blood (PB) or bone marrow (BM) blasts, more often EML, in particular lymphadenopathy, splenomegaly,

gingival hypertrophy and skin/mucosa involvement (Marcucci et al. 2005; Appelbaum et al. 2006). Based on the individual patient data-based meta-analysis performed by Schlenk et al. (2004) trisomy 22 was a significant prognostic variable for longer RFS. These findings have been confirmed by the CALGB showing a lower cumulative incidence of relapse (CIR) for patients with additional trisomy 22 compared to patients with inv(16) as a sole abnormality (Marcucci et al. 2005).

1.2.1.2.2. t(8;21)-positive AML

AML with the t(8;21) is associated with the M2 subtype and accounts for 5% to 12% of cases of AML. t(8;21) AML is frequently associated with specific characteristics, such as myeloid precursors containing Auer rods (Bloomfield et al. 1987), immunophenotypic aberrant expression of the CD19 (Kita et al. 1992) and the CD56 antigen (Reuss-Borst et al. 1994) and, in some patients, by extramedullary disease (EML) (granulocytic sarcomas) (Bloomfield et al. 1987; Tallman et al. 1993).

Additional cytogenetic, immunophenotypic and clinical features have been found in t(8;21) and some of them appear to have a prognostic impact on patients' outcome. In a recent individual patient data-based meta-analysis (Schlenk et al. 2004) performed on 392 adults with CBF AML significant prognostic variables for longer relapse free survival (RFS) and overall survival (OS) were lower WBC and higher platelet counts in the t(8;21) group. In addition, loss of the Y chromosome in male patients was prognostic for shorter OS. For patients who experienced relapse, second CR rate was significantly lower in patients with t(8;21), resulting in a significantly inferior survival after relapse compared to patients with inv(16). In analogy to inv(16)-assocaited leukemias these data have been confirmed in a study by the Cancer and Leukemia associated Group B (CALGB) (Marcucci et al. 2005). In this study a possible interaction between secondary chromosome abnormalities and race in patients with t(8;21) was observed. Finally, immunophenotypic expression of the neural cell adhesion molecule (NCAM) CD56 appears to be associated with an inferior outcome in CBF leukemia (Baer et al. 1997; Raspadori et al. 2001).

1.2.1.3. Treatment of primary CBF AML

In almost all studies of adult AML, CBF leukemia has been associated with a very high CR rate (approximately 90%) and the longest disease-free survival (DFS) at 5 years (approximately 50%), especially after dose-intensified cytarabine-based postremission therapy (Mrózek et al. 1997). Most important data came from the CALGB trial demonstrating that the cure rate can be improved with 3 or 4 cycles of intermediate-dose (IDAC) or high-dose cytarabine (HiDAC) as opposed to 1 cycle (Bloomfield et al. 1998; Byrd et al. 2002). In the study by Schlenk et al. (2004) for postremission therapy, intention-to-treat analysis revealed no difference between intensive HiDAC-based chemotherapy and autologous transplantation in the t(8;21) group as well as no difference between chemotherapy, autologous, and allogeneic transplantation in the inv(16) group, which is consistent with the data from Medical Research Council's 10th AML trial (MRC AML 10) (Grimwade et al. 1998), and French AML Intergroup (Delaunay et al. 2003).

Despite the fact that CBF leukemias are considered as "favorable/low-risk" with 5-year survival probabilities of 60% to 70%, RFS is worse with 40% to 50% of patients relapsing and eventually die from their disease (Schlenk et al. 2004; Marcucci et al. 2005). At this point the identification of these "high-risk patients" by genetic profiling becomes important. In addition, the identification of novel molecular markers might improve clinical outcome by using molecular targeted therapies as it has been recently demonstrated for *FLT3* mutated patients with normal karyotype AML.

1.3. Mutation classes in AML

The genetic events underlying AML pathogenesis appear to fall into two broadly defined complementation groups (Kelly et al. 2001): class I mutations that activate signal transduction pathways, resulting in enhanced proliferation and/or survival of hematopoietic progenitors and class II mutations affecting transcription factors or components of the transcriptional co-activation complex, resulting in impaired hematopoietic differentiation and/or aberrant acquisition of self-renewal properties by hematopoietic progenitors (Figure 2) (Speck et al. 2002).

In the model of Kelly and Gilliland both class I and class II mutations are required for the development of the leukemic phenotype that is characterized by proliferation and impaired differentiation of hematopoietic progenitors.

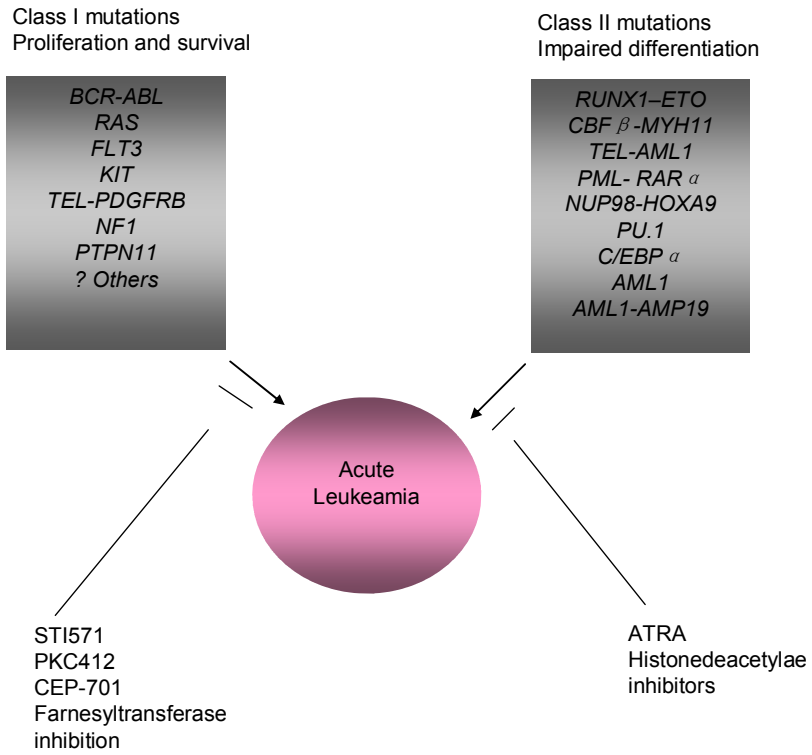

Figure 2. The model of cooperating mutations in acute leukemia (Speck et al. 2002). ATRA: all-*trans*retinoic acid; *CBFB*: core-binding factor β; CEP: anti-cancer compound; *PDGFRB*: platelet-derived growth factor receptor β; *PML*: promyelocytic leukaemia; *RARA*: retinoid-acid receptor; STI571: signal transduction inhibitor number 571, Gleevec; PKC: Protein kinase C.

1.3.1. Class I mutations

Prominent examples for class I mutations are mutations in the genes belonging to the Class III receptor tyrosine kinase (*RTKs*) family, like the *FMS* (Coussens et al. 1986), *KIT* (Yarden et al. 1987), *FLT3* (Rosnet et al. 1993a), *PDGFRa* (Claesson-Welsh et al. 1989) and *PDGFRb* genes (Yarden et al. 1986). Class III *RTK* genes share structural characteristics with 5 immunoglobulin-like domains (Matthews et al. 1991), a single transmembrane domain (TM), a juxtamembrane (JM) domain, 2 intracellular tyrosine kinase domains (TK1 and TK2) separated by a kinase insert (KI) domain, and a C-terminal domain in intracellular regions (Figure 3) (Yarden et al. 1987). In addition, they share common downstream affectors [*RAS*, *JAK/STAT* (Janus kinase/signal transducer and activator of transcription)] that have emerged as significant components in the

pathogenesis of AML (Weiner et al. 2000; Stephenson et al. 2001).

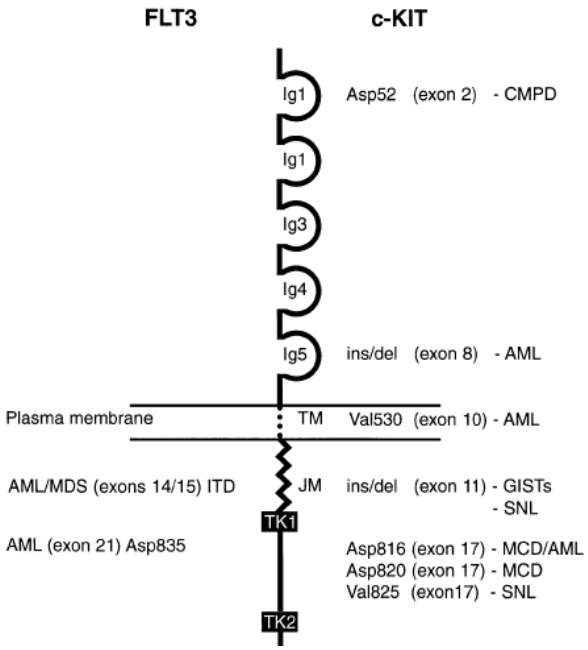

Figure 3. Cartoon of class III *RTK* (Yarden et al. 1987). A schematic of a canonical class III *RTK* monomer spanning the plasma membrane demonstrating five immunoglobulin-like domains (Ig1–5) in the extracellular ligand binding region, a single TM, a JM, two intracellular TK1 and TK2 divided by a KI. The position of reported mutations and their disease associations for *FLT3* and *KIT* are highlighted. AML: acute myeloid leukemia; CMPD: chronic myeloproliferative diseases; GISTs: gastrointestinal stromal tumors; SNL: sinonasal lymphomas; MCD: mast cell disease; MDS: Myelodysplastic syndrome; ins/del: insertion–deletion mutations.

1.3.1.1. Candidates for class I gene mutations in CBF AML

1.3.1.1.1. *RAS*

RAS oncogenes encode a family of membrane-associated proteins that regulate signal transduction on binding to a variety of membrane receptors. They play important roles in the regulation of cellular proliferation, the cell cycle and apoptosis (Byrne et al. 1998). In general, *RAS* mutations lead to a resistance of the *RAS* proteins to signaling provided by GTPase activating proteins thus remaining in the active GTP-bound state (Ahmadian et al. 1999; Seeburg et al. 1984). This constitutive activity is further translated via a cascade of key signaling events that contribute to the regulation of proliferation, apoptosis, and differentiation. There are 3 functional *RAS g*enes — *NRAS*, *KRAS*, and *HRAS* — and all homologs carry

mutations nearly exclusively in codons 12, 13, and 61, conferring constitutive activation of the *RAS* protein, which subsequently is held in GTP-bound status.

Different *RAS* oncogenes are preferentially associated with different types of human cancer (Kiyoi et al.1999; Beaupre et al. 1999). For example, *KRAS* mutations are predominantly associated with pancreatic, lung, and colon cancers. In myeloid malignancies, *NRAS* mutations occur in varying frequencies (11 - 40%) more frequently than *KRAS* mutations (Bowen et al. 2005; Boissel et al. 2006; Bacher et al. 2006), whereas *HRAS* mutations are rare (Bos JL.1989). In CBF AML, *NRAS* mutations have been reported in approximately 13 - 38% of cases (Panagopoulos et al. 1996; Kiyoi et al. 1999; Bacher et al. 2006); whereas *KRAS* mutations vary from 2 - 23% (Kiyoi et al. 1999; Bacher et al. 2006; Boissel et al. 2006). To date, the prognostic impact of *RAS* mutation is still under discussion and seems to depend on the type of the disease (Kiyoi et al.1999; Stirewalt et al. 2001; Ritter et al. 2004)

1.3.1.1.2. *KIT*

Human *c-KIT* (CD117), the cellular counterpart of *v-KIT,* derived from the Hardy–Zuckerman 4-feline sarcoma virus (Besmer et al. 1986), encodes a glycoprotein receptor that binds stem cell factor (SCF, kit ligand, mast cell growth factor). *KIT* is located in tandem on the chromosomal region 4q11-q13 (Gronwald et al. 1990; Giebel et al. 1992). At the genomic level *KIT* spans more than 70 kb and includes 21 exons (Vandenbark et al. 1992). *KIT* receptor is a member of the type III receptor tyrosine kinase family, which also includes *FLT3* and *PDGFRs* (Heinrich et al. 2002). Activating mutations in *KIT* were first described in the mast cell leukemia cell line HMC-1 and activating mutations in *KIT* have subsequently been identified in AML, mastocytosis (Nagata et al. 1995; Longley et al. 1999), GIST and seminoma/dysgerminoma (Hirota et al. 1998; Kemmer et al. 2004). Functional studies demonstrated that these *KIT* mutations conferred cytokine-independent survival and a growth advantage to cells (Kitayama et al. 1996). Ligand-independent activation of *KIT* can be caused by gain-of-function mutations that have been reported in between 12.8% and 48.1% of adult CBF AML (Wang et al. 2005; Schnittger et al. 2006; Paschka et al. 2006). The prognostic significance of *KIT* mutations in patients with CBF has been analyzed by several groups

revealing inconsistent results (Care et al. 2003; Cairoli et al. 2006; Paschka et al. 2006). However, more recent data published by the CALGB in a large patient cohort (Paschka et al. 2006) demonstrated that *KIT* mutations are associated with a significantly adverse OS probability.

1.3.1.1.3. *FLT3*

The human *FLT3* gene maps to chromosomal band 13q12 and is closely linked, in a head-to-tail fashion, to *FLT1*, a class V *RTK* (characterized by seven immunoglobulin-like domains) (Imbert et al. 1994). Recently, *FLT3* gene has been shown to comprise 24, rather than the expected 21 exons typical of other class III *RTKs*, with seven instead of four exons encoding the first three immunoglobulin-like repeats (Abu-Duhier et al. 2001). *FLT3* is normally expressed by hematopoietic stem/progenitor cells (HSPCs) and expression is lost as hematopoietic cells differentiate. A large body of work has shown that *FLT3* plays a role in survival, proliferation and differentiation of hematopoeitic cells (Gilliland et al. 2002; Stirewalt et al. 2003).

Activation of *FLT3* by mutation results in autophosphorylation as well as phosphorylation of a number of other proteins, either directly or indirectly. Examination of these proteins clearly demonstrated that the major signal transduction pathways leading from *FLT3* include *PI3*-kinase/*AKT*, *RAS/MAPK*, and *STAT5* (Small D. 2006). *FLT3* mutations are one of the most frequent somatic alterations in AML, occurring in approximately 25% to 30% of patients. *FLT3* mutations consist of two major types: internal tandem duplication (ITD) mutations of 3 - 400 base pair (bp) (always in frame) that map to the juxtamembrane region (23% of AML patients) and point mutations that most frequently involve aspartic acid 835 of the tyrosine kinase domain (TKD) but have also been found less frequently in several other sites (8 - 12% of AML patients) (Yamamoto et al. 2001). Recently, Schessl et al. (2005) showed that *RUNX1-CBFA2T1* and the *FLT3*-ITD mutation collaborate in inducing acute leukemia in a murine BM transplantation model. Moreover, they identified additional *FLT3*-ITD, *KIT* and *NRAS* mutations in 28% of 135 t(8;21)-positive leukemias supporting the concept of oncogenic cooperation between *RUNX1-CBFA2T1* and activating gene mutations. Recently, several study groups reported a substantial proportion of CBF AML patients

carrying *FLT3*-ITD up to 9%, as well as TKD mutations in 2 - 7% (Yamamoto et al. 2001; Care et al. 2003; Boissel et al. 2006). Some studies have shown that patients with *FLT3*-ITD have a distinctly different gene expression profile than those with *FLT3*-TKD (Neben et al. 2005). Other studies have defined signal transduction differences in in vitro and in vivo model systems (Choudhary et al. 2005; Grundler et al. 2005), substantiating that biologic differences underlie the differences in clinical outcome in patients with the 2 distinct *FLT3* mutations. *FLT3*-ITD mutations have been shown to be associated with a poor prognosis for both pediatric and adult patients, whereas in a very recent study on a large patient population the patients with *FLT3*-TKD point mutations had an improved outcome (Mead et al. 2007).

1.3.1.1.4. *JAK2*

JAK2 is a tyrosine kinase involved in the transduction of cellular growth stimuli (Witthuhn et al. 1993; Parganas et al. 1998). In vitro experiments revealed that hematopoetic cells transduced with the *JAK2* V617F mutation, that has recently been identified, showed cytokine independent growth and activation of the *STAT* and extracellular receptor kinase signal transduction pathways. Based on that findings showing that the *JAK2* V617F mutation mediates increased activity of the *JAK/STAT* pathway resulting in increased cell proliferation and survival (Kralovics et al. 2005), *JAK2* mutations is considered as a class I mutation within the model of two complemenation groups of mutations in AML.

The *JAK2* V617F mutation has recently been discovered as a single-site, clonal, gain-of-function mutation in myeloid cells from the majority of patients with chronic myeloproliferative disorders (MPD) (James et al. 2005), whereas in AML the *JAK2* V617F mutation is rare (Levine et al. 2005; Fröhling et al. 2006). Lee et al. (2006) reported *JAK2* mutations were found in only 3/113 AML patients, however, two of three cases exhibiting *JAK2* V617F were diagnosed as t(8;21)-positive AML. In addition, in the study from Döhner et al. (2006) *JAK2* V617F mutations were identified in 6% of t(8;21)-positive AML whereas none of the inv(16)/t(16;16)-positive cases had this type of mutation. These findings together with the observation from Schessl et al. (2005) sustain the model of cooperating gene mutations.

1.3.2. Class II mutations

The second broad complementation group comprises mutations in transcription factors or transcriptional co-activators that are important for differentiation of hematopoietic cells (Gilliland et al. 2004). These include the CBF transcription complex and *CEBPα*, as well as *RARA* (Sirulnik et al. 2003), *MLL* (Armstrong et al. 2003), and components of the transcriptional co-activation complex such as *CBP*, *MOZ*, and *TIF2* (Borrow et al. 1996; Liang et al. 1998). Gene rearrangements involving these proteins also play an important role in activation of pathways responsible for self-renewal, such as the canonical *WNTCTNNB1*, *NOTCH*, and *BMI1* pathways (Jordan et al. 2004; Huntly et al. 2005). These pathways are likely to be critical for generation of leukemic stem cells that are ultimately responsible for the maintenance of the leukemic phenotype. Of these, the most extensively investigated are *AML1-ETO* and *CBFβ-MYH11* fusions. Homozygous loss of function of either *AML1* or *CBFβ* in genetically engineered mice results in a complete lack of definitive hematopoiesis, indicating that both components of CBF are essential for hematopoietic development (Gilliland et al. 2004). The molecular biology characteristic of *AML1-ETO* and *CBFβ-MYH11* were already discussed in Section 1.2.1. In recent years many groups investigated the mechanisms by which the CBFs and their partner fusion genes contribute to the pathogenesis of AML. However, studies from murine models clearly demonstrated that the neither the fusion gene *AML1-ETO* nor *CBFβ-MYH11* are sufficient to cause an AML phenotype (Castilla et al. 1999; Schessl et al. 2005).

The observation that *AML1-ETO* as a single factor is nonleukemogenic is further supported by findings that nonleukemic *AML1-ETO*–expressing progenitor cells can be isolated from healthy individuals as well as AML patients in remission, which suggests that additional mutations in these *AML1-ETO*–positive progenitors are necessary for the transformation into leukemia-initiating cells (Miyamoto et al. 2000; Reya et al. 2001). The importance of collaborating genetic events in the pathogenesis of *AML1-ETO*–positive leukemias has indeed been shown in different murine models. As already discussed, in the study by Schessl et al (2005), in a murine BM transplantation model, *AML1-ETO* collaborates with *FLT3*-ITD in inducing acute leukemia. The need for additional genetic events for full transformation has also been demonstrated for *CBFβ-MYH11*, for example, a

mouse "knock-in" model created by inserting the *CBFβ-MYH11* fusion gene into the mouse *CBFβ* locus in embryonic stem cells, failed to develop leukemia at high frequency within the first year of life (Castilla et al. 1996). However, after exposure to N-ethyl-N-nitorsourea (EUN), a DNA-alkylating agent, 4-16 week-old chimaeras developed leukemia, with some features of inv(16)-positive AML after several months. Furthermore, co-expression of *CBFβ-MYH11* with the human papilloma virus (HPV) E7 oncogene, or expression of the fusion protein in the absence of the tumor suppressor genes p16INK4a and p19 ARF leads to acute leukemia (Yang et al. 2002)

In addition, several groups have reported some AML patients in long-term remission retain the *AML1-ETO* fusion gene in a small, but stable, fraction of their bone marrow cells (Nucifora et al. 1993), while some patients persisted of chimaeric *CBFβ-MYH11* transcripts even after allgeneic bone marrow transplantation (Tobal et al. 1995; Costello et al. 1997b). In addition, Wiemels et al. (2002) detected *AML1-ETO* in Guthrie blood spots of children who developed a corresponding AML many years later, while the prenatal origin of childhood leukaemia harboring *CBFβ-MYH11* has been reported with postnatal latencies of approximately 10 years (McHale et al. 2003). The long latency periods have been interpreted to reflect postnatal persistence of translocation-positive, quiescent multi-potent cells, which, upon later recruitment into the myeloid differentiation pathway, acquire additional secondary changes necessary for leukaemia (Reilly JT. 2004). Taken together, these experimental and clinical studies strongly support the model of two complementation groups of mutations in CBF leukemia harboring the specific fusion genes in potential leukemic precursor cells that might be transformed by additional secondary genetic events.

1.4. The aim of the study

Based on the findings of recent studies implicating a model of two complementation groups of mutations for the development of AML, the aims of this study are:

1) to evaluate the incidence of *RAS* (*NRAS*/*KRAS* exon 1, exon 2), *KIT* (exons 8, 10, 11, and 17), *FLT3* (ITD and TKD) and *JAK2* (V617F) mutations in a large cohort of inv(16)/t(16;16)-positive and t(8;21)-positive AML patients enrolled

in 5 treatment trials of the AML Study Group (AMLSG) and the Hemato-Oncologie voor Volwassenen Nederland (HOVON) study.

2) to evaluate the prognostic impact of each single gene mutation on clinical outcome and to examine potential interaction of different gene mutations.

3) to provide a rationale for the treatment with molecular-targeted therapies such as tyrosine kinase inhibitors in patients exhibiting one of these gene mutations.

2. Material and Methods

2.1. Material

2.1.1. DNA extraction

Chemicals

DNAzol reagent	GibcoBRL, Eggenstein, Germany	
Ethanol	Riedel-de Haën, Seelze, Germany	100%
EB buffer	Qiagen, Hilden, Germany	
EDTA	Merck, Darmstadt, Germany	
Tris-HCl	Roth, Karlsruhe, Germany	

Buffer and solutions

Ethanol	100% ethanol 750mL add double distilled H_2O (ddH_2O) to final volume of 1 L	75% (v/v)
TE buffer	10 mM Tris-HCl (pH 8) 2 mM EDTA (pH 8) add ddH_2O to final volume of 1 L	pH 8 (v/v)
Tris-HCl	121.1 g Tris base add ddH_2O to volume of 850 mL, pH adjusted with HCl to 7.5	1M (w/v)

Instruments

Photometer	Eppendorf AG, Hamburg, Germany
Eppendorf centrifuge 5415D	Eppendorf AG, Hamburg, Germany

2.1.2. Polymerase chain reaction (PCR)

Enzymes and chemicals

AmpliTaq Gold polymerase	Applied Biosystems, Weiterstadt, Germany
10 x buffer 3	New England Biolabs, Beverly, MA
dNTPs	Roche, Mannheim, Germany
EcoR V	Roche, Mannheim, Germany
Hot Star DNA Polymerase	Qiagen, Hilden, Germany
Taq DNA polymerase	Applied Biosystems, Weiterstadt,

	Germany	

Primers and dNTPs preparation

dNTPs for standard PCR	12.5 µL of 100 mM stock solution of each dNTP, add ddH_2O to final volume of 1 mL	100 mM (v/v)
Primer for standard PCR	100 µL of 100 mM stock solution of primer, add ddH_2O to final volume of 1 mL	10 mM (v/v)

Instrument

Thermocycler (GeneAmp PCR system 2700)	Applied Biosystems, Singapore

2.1.2.1. Synthetic oligonucleotide primers

All oligonucleotide primers were purchased from Roche, Mannheim, Germany. The following nucleotides were used.

Table 4. Oligonucleotide primers used for mutation screening

Gene	Exon	Sequence (5'- 3')	Fragment size (bp)
NRAS	1	Forward: CAC TAG GGT TTT CAT TTC CAT TG Reverse: TCC TTT AAT ACA GAA TAT GGG TAA AGA	300
	2	Forward: AA GTG GTT ATA GAT GGT GAA ACC Reverse: AG ATC ATC CTT TCA GAG AAA ATA AT	201
KRAS	1	Forward: GCC TGC TGA AAA TGA CTG AA Reverse: GT CCT GCA CCA GTA ATA TGC	162
	2	Forward: TT TTG AAG TAA AAG GTG CAC TG Reverse:TGG CAA ATA CAC AAA GAA AGC C	195
KIT	8	Forward: GCT GAG GTT TTC CAG CAC TC Reverse: AAT TGC AGT CCT TCC CCT CT	219
	10	Forward: CCA AGG TGAA GCT CTG AGA C Reverse: CAC TGT ACT TCA TAC ATG GG	366
	11	Forward: CCA GAG TGC TCT AAT GAC TG Reverse: GGA AGC CAC TGG AGT TCC TT	274
	17	Forward: TGT ATT CAC AGA GAC TTG GC Reverse: GGA TTT ACA TTA TGA AAG TCA CAG G	218
FLT3	14/15	Forward: GCA ATT TAG GTA TGA AAG CCA GC Reverse: CTT TCA GCA TTT TGA CGG CAA CC	328
	20	Forward: CCA GGA ACG TGC TTG TCA Reverse: TCA AAA ATG CAC CAC AGT GAG	196
JAK2	12	Forward 1: AGC ATT TGG TTT TAA ATT ATG GAG TAT ATT (sepecific) Reverse: CTG AAT AGT CCT ACA GTG TTT TCA GTT TCA	203

12	Forward 2: ATC TAT AGT CAT GCT GAA AGT AGG AGA AAG (internal control) Reverse: CTG AAT AGT CCT ACA GTG TTT TCA GTT TCA	364

2.1.3. Agarose gel electrophoresis

Chemicals

Agarose	Sigma, St. Louis, USA	
DNA-ladder	Invitrogen, Carlsbad, CA, USA	100 bp
Loading buffer	Invitrogen, Carlsbad, CA, USA	
Ethidium bromide	Carl Roth GmbH, Mannheim, Germany	10 mg/mL

Buffer and solutions

Agarose gel	12 g agarose	4% (w/v)
	300 mL 1 X TAE buffer	
50 X TAE (Stock solution)	242 g Tris base	w/v
	57.1 mL acetic acid	
	0.5 M 100 mL EDTA	
1 X TAE (Electrophoresis buffer)	50 mL 50 X TAE stock solution	v/v
	add ddH_2O to final volume of 5 L	

Instruments

Electrophoresis apparatus	Owl Separation System Incorporation, Portsmouth, NH, USA
Electrophoreses power supply	Amersham Pharmacia Biotech, Upsala
UV light	Schweden Transilluminator, UVP, Upland, USA
Video camera	MWG Biotech, Ebersberg, Germany

2.1.4. Denaturing high performance liquid chromatography (DHPLC)

Buffers and solutions

DNAsep® Wash Solution	Transgenomic, Omaha, NE, USA
WAVE High-Range Mutation Standard	Transgenomic, Omaha, NE, USA
WAVE Low-Range Mutation Standard	Transgenomic, Omaha, NE, USA
WAVE Optimized® TEAA Buffer A	Transgenomic, Omaha, NE, USA
WAVE Optimized® TEAA Buffer B	Transgenomic, Omaha, NE, USA
WAVE Optimized® Solution D	Transgenomic, Omaha, NE, USA

WAVE Optimized® Syringe Wash Solution	Transgenomic, Omaha, NE, USA

Instruments and software

DNAsep® Cartridge	Transgenomic, Omaha, NE, USA
FCW-200 in-line fragment	Transgenomic, Omaha, NE, USA
Navigator software 4.1.42	Transgenomic, Omaha, NE, USA
3500HT WAVE DNA Fragment Analysis System	Transgenomic, Omaha, NE, USA

2.1.5. Purification of PCR products

Name

Isopropanol (70%)	Merck, Darmstadt, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany

2.1.6. Cycle sequencing reaction (CSR)

ABI PRISM Big Dye Terminator Cycle Sequencing Kit was purchased from Applied Biosystems, Darmstadt, Germany.

2.1.7. Dye removal

DyeEx 2.0 Spin Kit was purchased from Qiagen, Hilden, Germany.

2.1.8. Direct DNA sequencing

Name

ABI-PRISM310 genetic analyzer	Applied Biosystems, Foster City, CA
Data Collection Software v1.1	Applied Biosystems, Foster City, CA
Heat block	Grant Instruments Ltd, Cambridge, England

2.2. Patients

A total of 167 inv(16) (n = 101) and t(8;21) (n = 66) positive AML-patients were included in the present study. One hundred sixty four patients were treated within one of the AMLSG treatment trials [AML HD93 (age 16 - 60 years), August 1993 to

January 1998; AML HD98-A (age 18 - 60 years) and AML HD98-B (age ≥ 60 years), February 1998 to April 2004; or AMLSG 07-04 (age 18-60 years), and AMLSG 06-04 (age ≥ 60 years, both ongoing]. Three patients were included in the HOVON treatment trial (age ≥ 60 years, October 2000 to November 2004). Among all analyzed patients, 94 younger inv(16)-positive patients (median age 40 years, range from 18-58 years) were enrolled in three multicenter treatment trials [AML HD93 (n = 21), AML HD98-A (n = 59), AMLSG 07-04 (n = 14)], and 52 younger t(8;21)-positive patients (median age 42 years, range from 18-59 years) entered into the same treatment protocols [AML HD93 (n = 7), AML HD98-A (n = 37), and AMLSG 07-04 (n = 7)]. A detailed distribution of patient numbers enrolled in above treatment trials is given in Table 5. All patients included were diagnosed according to FAB Cooperative Group criteria (Bennett et al. 1985) and gave informed consent. Institutional review board approval was provided from all participating centers.

Table 5. Distribution of patient numbers enrolled in various AMLSG trials at diagnosis and number of patients per postremission therapy in first CR.

		inv(16)				t(8;21)			
	Treatment trial	**Total**	**Chemo**	**Auto**	**Allo**	**Total**	**Chemo**	**Auto**	**Allo**
	AML HD93	21	12	0	2	7	5	0	2
< 60years	AML HD98-A	59	20	23	10	39	31	1	1
	AMLSG 07-04	14	13	0	0	7	3	1	1
	AML HD98-B	6	4	0	0	9	5	0	0
≥ 60years	AMLSG 06-04	1	1	0	0	1	1	0	0
	HOVON	0	0	0	0	3	3	0	0

AML: acute myeloid leukemia; AMLSG: AML Study Group; Chemo, chemotherapy; Auto, autologous transplantation; Allo, allogeneic transplantation.

De novo AML was diagnosed in 93 patients with inv(16) and 47 patients with t(8;21), respectively; among them 6 and 11 patients had therapy-related AML/secondary AML (t-AML/s-AML), and in 2 and 8 patients the information was missing. For the present study, the inclusion criteria used were as follows: (1) presence of t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22) on standard karyotypic analysis or presence of the *RUNX1-CBFA2T1* or the *CBFß-MYH11* fusion gene by molecular screening, (2) availability of BM or PB samples at diagnosis. All patients gave informed consent for both treatment and

cryopreservation of BM and PB according to the Declaration of Helsinki.

2.3. Treatment

All younger patients (16 to 60 years) received intensive, response-adapted double-induction and consolidation therapy (Figure 4). In AML HD93 and AML HD98-A, double-induction therapy consisted of a course of idarubicin 12 mg/m^2 on days 1, 3, and 5; cytarabine 100 mg/m^2 continuously on days 1 through 7; and etoposide 100 mg/m^2 on days 1 through 3 (ICE), followed by a second course of ICE started between days 21 and 28 in patients with a response to the first course, or by a course of a high-dose cytarabine (HAM)-base (3 g/m^2 of cytarabine every 12 hours on days 1 through 3; 12 mg/m^2 of mitoxantrone on days 2 and 3) regimen in patients with ICE-refractory disease. Response assessment was scheduled on day 21 of first induction therapy and after hematologic reconstitution after second induction therapy. First consolidation therapy with one course of HAM (cytarabine 3 g/m^2 every 12 hours on days 1 through 3; mitoxantrone 12 mg/m^2 on days 2 and 3). For further postremission therapy differed between trials (Figure 4). In the AML HD93 trial, all patients with CBF-AML were assigned to a second course of HAM. In the AML HD98-A trial, patients exhibiting the t(8;21) were assigned to a second course of HAM, whereas patients exhibiting inv(16) were assigned to autologous or allogeneic stem cell transplantation (SCT), if an HLA-compatible family donor was available. In AMLSG 07-04 trial, the same double-induction ICE regime with aforementioned two studies trials with or without the integration of all-*trans*retinoic acid and/or Valproic acid [ATRA: 45 mg/m^2 on days 3 through 5 and 15 mg/m^2 on days 6 through 28; 400 mg Valproic acid on days 1, 2, subsequently p.o. plasma-level adapted, aspired plasma-level 100 mg/L (60 -150 mg/L)] as adjunct to the induction therapy. Response assessment was scheduled on day 15, 21, and 28 of first induction therapy and after hematologic reconstitution after second induction therapy. Three cycles consolidation therapy with HiDAC (3 g/m^2 of cytarabine every 12 hours on days 1 through 3) were administered with or without the integration of ATRA and/or valproic acid (ATRA; 15 mg/m^2 on days 6 through 21; 400 mg Valproic acid, aspired plasma-level 100 mg/L). Response evaluation was performed to every patient, before start of the following 2nd and 3rd consolidation cycles. Only patients in continuous CR or CRi (CR with incomplete hematological recovery) qualify for the following cycles.

CBF patients ≥ 60 years were enrolled in the AML HD98-B and AMLSG 06-04, respectively. In the AML HD98-B trial, randomized induction therapy consisted of 2 courses of 12 mg/m^2 idarubicin on days 1 and 3, 100 mg/m^2 cytarabine continuously on days 1 through 5, and 100 mg/m^2 etoposide on days 1 and 3 (ICE) with or without ATRA (45 mg/m^2 on days 3 through 5 and 15 mg/m^2 on days 6 through 28), followed by first consolidation therapy with a course of 0.5 g/m^2 cytarabine every 12 hours on days 1 through 3 and 10 mg/m^2 mitoxantrone on days 2 and 3 (HAM) with or without ATRA (15 mg/m^2 on days 3 through 28). For further postremission therapy, patients were randomized to intensive second consolidation therapy (12 mg/m^2 idarubicin on days 1 and 3, 100 mg/m^2 etoposide on days 1 through 5) or 12 monthly courses of outpatient maintenance therapy (5 mg idarubicin orally on days 1, 4, 7, 10, and 13 and 100 mg etoposide orally on days 1 and 13) (Figure 4). Patients not responding to the first course of induction therapy were assigned to receive a course of 0.5 g/m^2 cytarabine every 12 hours on days 1 through 3, 250 mg/m^2 etoposide continuously on days 4 and 5, 45 mg/m^2 ATRA on days 3 through 5, and 15 mg/m^2 ATRA on days 6 through 28 (A-HAE), followed by a course of A-HAM and 6 monthly courses of outpatient maintenance therapy (5 mg idarubicin on days 1, 4, 7, 10, and 13 and 15 mg/m^2 ATRA on days 1 through 28). In AMLSG 06-04 trial, the same dosage and schedule double induction therapy as AML HD98-B treatment consisted of idarubicin and cytarabine (IC) with ATRA (45 mg/m^2 on days 4 through 6 and 15 mg/m^2 on days 7 through 28), followed by first consolidation therapy with a course of HAM with ATRA (15 mg/m^2 on days 4 through 28). For further postremission therapy, patients were assigned to intensive second consolidation therapy idarubicin and etoposide (IE) with ATRA (15 mg/m^2 on days 4 through 28). During the whole double induction and consolidation therapy, patients with or without Valproic acid were assigned [Valproic acid, plasma-level adapted, aspired plasma-level 100 mg/L (60 - 150 mg/L)]. Patients enrolled in the HOVON study protocol received HiDAC.

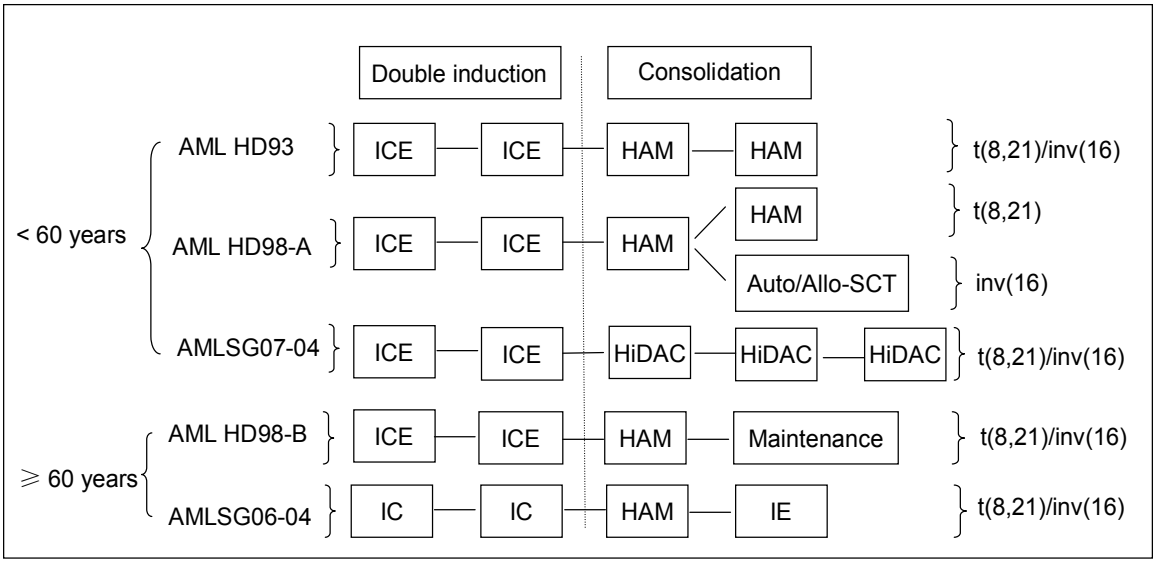

Figure 4. Summary of AMLSG treatment protocols. AMLSG: AML Study Group; ICE: idarubicin, cytarabine, etoposide; HAM: high-dose cytarabine and mitoxantrone; HiDAC: high-dose cytarabine; Auto-SCT: autologous stem-cell transplantation; Allo-SCT: allogeneic stem-cell transplantation; IC: idarubicin, cytarabine; IE: idarubicin, etoposide.

2.4. Cytogenetic and molecular genetic analysis

Pretreatment samples from all patients were analyzed centrally by G-banding analysis and fluorescence *in situ* hybridization (FISH). Conventional cytogenetic studies were performed using standard techniques, and chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (Mitelman et al. 1995). To improve the accuracy of cytogenetic diagnosis, all specimens were also analyzed by *FISH* using a comprehensive DNA probe set allowing for the detection of the most relevant AML-associated genomic aberrations (Fröhling et al. 2002).

2.5. Analysis of *RAS*, *KIT*, *FLT3* and *JAK2* gene mutations

2.5.1. DNA extraction

Genomic DNA from enriched mononuclear cells was extracted using DNAzol reagent. For lysis, 2 mL of DNAzol was added to $1X10^7$ cells, and 2 mL of 100% ethanol was added to precipitate DNA. DNA was transferred to a clean tube and centrifuged for 15 minutes (min.) at 13,000 rpm at 4°C. The samples were then washed twice with 75% ethanol and the DNA was finally dissolved in 200 - 300 µL

water or TE buffer. Determination of the DNA concentration was performed with a 1:50 dilution of DNA in ddH_2O and absorbency was measured 260 and 280 nm using a photometer. Since a solution with an OD260 of 1 contains approximately 50 µg DNA per milliliter, the DNA concentration in a sample was as follows:

DNA concentration = OD260 X dilution factor X 50 µg/mL

2.5.2. Polymerase chain reaction (PCR)

2.5.2.1. Amplification of *NRAS*, *KRAS* and *KIT*

The amplification of *NRAS* and *KRAS* (exons 1, 2), respectively, as well as *KIT* exons 8, 10, 11 and 17 were performed using conventional genomic DNA PCR. Primer pairs of all target genes are listed in Table 4. Briefly, the total reaction volume of 50 µL contained 50 ng template DNA, 10 pmol of each primer, deoxynucleotide triphosphate (dNTPs, 10 mmol/L each), 5 µL of 10 X High fidelity buffer, and 2 µL of 25 mM $MgSO_4$, 2.5 U proofreading DNA Optimase polymerase. Samples were amplified using the following cycling conditions: denaturation step at 95°C for 5 min.; followed by 35 cycles at 94°C for 30 sec., 57°C for 1 min., 72°C for 1 min.; and a final extension step at 72°C for 10 min..

2.5.2.2. Amplification of *FLT3*-ITD

FLT3-ITDs were identified by amplification using conventional genomic DNA PCR. All primers used are shown in Table 4. The total reaction volume of 50 µL contained approximately 100 ng genomic DNA and 10 pmol each primer, 10 mM dNTP, 5 µL of 10 X PCR buffer with 15 mM $MgCl_2$, 10 µL Q-solution, 2.5 U Hot Star DNA Polymerase. Samples were amplified using standard PCR conditions (95°C for 10 min.; 35 cycles at 95°C for 1 min., 56°C for 1 min., 72°C for 2 min.; 72°C for 10 min.).

2.5.3. PCR with restriction fragment length polymorphism (PCR-RFLP)

2.5.3.1. Detection of *FLT3*-TKD

A PCR-RFLP assay described by Yamamoto et al. (2001) was performed to

detect *FLT3*-TKD (D835) mutations. Codons D835 and I836 are encoded by the nucleotide GATATC, which forms the *EcoRV* restriction site (Figure 5). Amplification was performed by genomic PCR using primers pairs as previously reported (Table 4) (Fröhling et al. 2002).

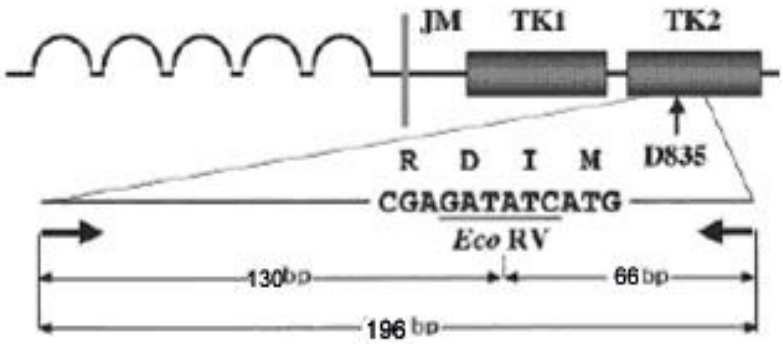

Figure 5. Detection of D835 mutations in the *FLT3* gene. The amplified products of wild type were digested to 2 bands (130 bp and 66 bp) by *EcoRV*. When amplified products contained D835 mutations, undigested bands (196 bp) were visualized on agarose gel electrophoresis.

The PCR reaction mixture contained 1 µL DNA template, 10 pmol of each primer, 10 mM dNTPs, 5 µL of 15 mM $MgCl_2$, 1.5 U AmpliTaq Gold polymerase and its optimum buffer in a total volume of 50 µL. Samples were amplified using standard PCR conditions (95°C for 10 min.; 35 cycles at 95°C for 1 min., 58°C for 1 min., 72°C for 1 min.; 72°C for 10 min.). PCR products were then digested with *EcoR V* (15 U/µL). Briefly, 50 µL PCR products were incubated with 6 µL of 10 x buffer 3, 2 µL *EcoRV* and 2 µL water in a total volume of 40 µL at 37°C for 2 hours. Subsequently, the fragments were resolved in a 4% agarose gel. Undigested bands of the amplified products were cut out from the gel, and directly sequenced.

2.5.4. Detection of *JAK2* mutations using allele-specific PCR

Allele-specific PCR was used to detect the presence of *JAK2* exon 12 specific mutations located at Val617 (Baxter et al. 2005). The first forward primer is specific for the mutant allele and contains an intentional mismatch at the third nucleotide from the 3' end to improve specificity (giving a 203 bp product); the second primer amplifies a 364 bp product from both mutant and wild-type alleles and serves as an internal PCR control.

100 ng of sample DNA was amplified using 10 pmol of a common reverse primer and 5 pmol of each forward primer (Table 4), 1 µL dNTP (10 mM each), 2.5

µL $MgCl_2$ (50 mM), 2.5 U Platinum Taq DNA polymerase and its optimum buffer. Reaction conditions for amplification was as follows: 95 °C for 5 min.; 36 cycles at 95 °C for 15 sec., 58 °C for 1 min. and 72 °C for 1 min.; followed by a final step at 72 °C for 10 min.

2.5.5. Agarose gel electrophoresis

To visualize amplified PCR products, agarose gel electrophoresis was performed. Briefly, 2 µL loading buffer was added to 8 µL PCR product and subsequently loaded to a 2% agarose gel in 1 x TAE buffer. A 100 bp DNA-ladder was used to as size marker. Electrophoresis was run by 120V/80mA for 1 hour. Subsequently, the gel was stained in 1 x TAE buffer containing 0.5 µg/mL ethidium bromide for 15 - 30 min. For documentation, DNA bands were visualized by UV light at 302 nm and photographed using a digital camera.

2.5.6. Isolation of the DNA fragments from agarose gels

After PCR amplification, DNA fragments were size-fractionated in agarose gels and the specific bands were cut out of the gel with a scalpel under UV light. DNA extraction was performed using the QIAquick Gel Extraction Kit according to the instructions of the manufacturer.

2.5.7. Denaturing high performance liquid chromatography (DHPLC)

DHPLC analysis on the Transgenomic WAVE® System by temperature modulated heteroduplex analysis offers a rapid, highly sensitive, automated and cost-effective method for mutation screening, and has been demonstrated to be more sensitive than both single-strand conformational polymorphism analysis and direct sequencing in the detection of germ-line and somatic mosaicism (Xiao et al. 2001).

DHPLC is based on the detection of heteroduplexes in short segments of DNA by ion-reverse phase high performance liquid chromatography (Xiao et al. 2001). Partial heat denaturation within an acetonitrile gradient leads to the separation of the DNA strands, resulting in the formation of hybrid wild-type/mutant heteroduplexes. These heteroduplexes have a reduced column retention time and hence an altered mobility compared to their homoduplex counterpart (Figure 6).

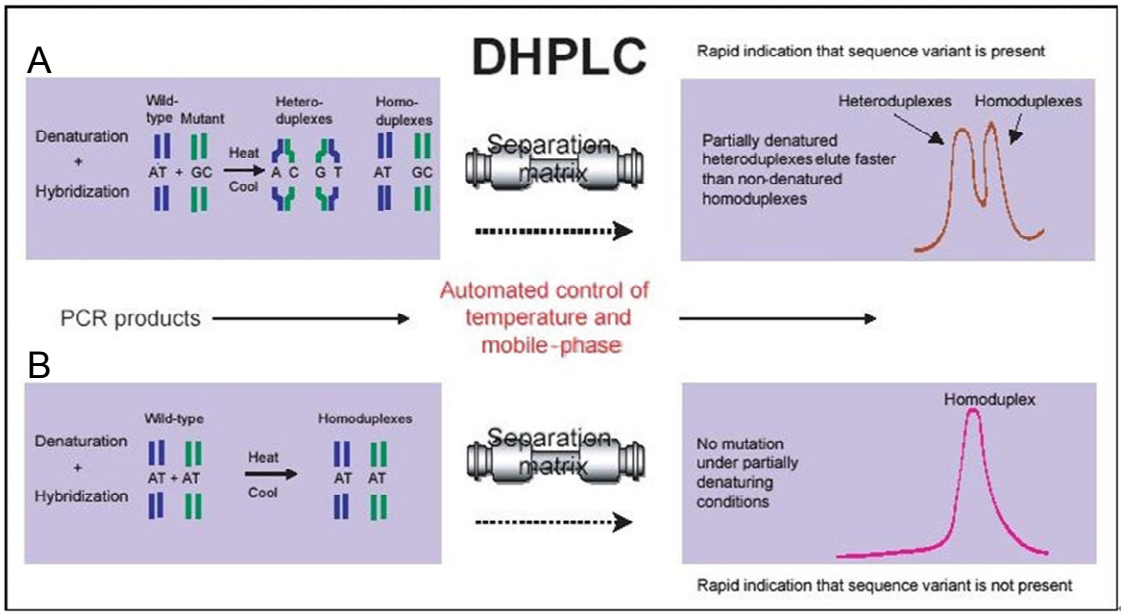

Figure 6. Principle of mutation detection by DHPLC. A: In the presence of mismatch, not only are the original homoduplices formed, but also the sense and antisense strands of either homoduplex form heteroduplices; B: Heteroduplices denature more extensively at the analysis temperature (ranges from 50°C to 70°C) and are eluted earlier than the homoduplices in the DNA Sep column. DHPLC: denaturing high performance liquid chromatography.

For detection of DNA sequence changes, DHPLC was performed using the 3500HT WAVE DNA Fragment Analysis System. Heteroduplex formation was created by denaturating the PCR products for 5 min. at 95°C and then allowing the samples to reanneal by decreasing the temperature 1°C per min. from 95°C to 45°C. Five to ten µL of the PCR products (*NRAS*, *KRA*S, and *KIT*) were run on the DHPLC via rapid mode with a gradient time of 2.5 min and a total run time of 3 min., in comparison to control samples run in parallel. The start ratio of buffers A [0.1M triethylammonium acetate (TEAA), 0.025% acetonitrile (ACN) (v/v), pH 7.0], and B [0.1M TEAA, 25% ACN (v/v), pH 7.0] was calculated using Navigator software (Transgenomics). The stop ratio was set as the buffer B start value + 12%. The elution temperatures for each amplicon were recommended by the Navigator software and then optimized by studying alterations in the elution profiles of the samples within a temperature varied according to the analyze products: *KIT* exon 8: 50°C; *KIT* exon 10: 57°C; *KIT* exon 11: 57°C; *KIT* exon 17: 56.7°C; *NRAS* exon 1: 59.8°C; *NRAS* exon 2: 58.2°C; *KRAS* exon 1: 58.2°C; *KRAS* exon 2 58.2°C. To identify homozygous mutations, equal aliquots of a known wild-type sample were mixed with the DNA prior to the re-annealing step to enable heteroduplex

formation.

Samples exhibiting an abnormal DHPLC profile (Figure 7) were subsequently sequenced to confirm the mutation. In case, sequencing was insufficiently sensitive to confirm mutation, fragment collection was carried out using a Transgenomic FCW-200 in-line fragment collector (Emmerson et al. 2003). Collected positive fractions served as a template for re-amplification, which were subsequently directly sequenced to reveal the mutation.

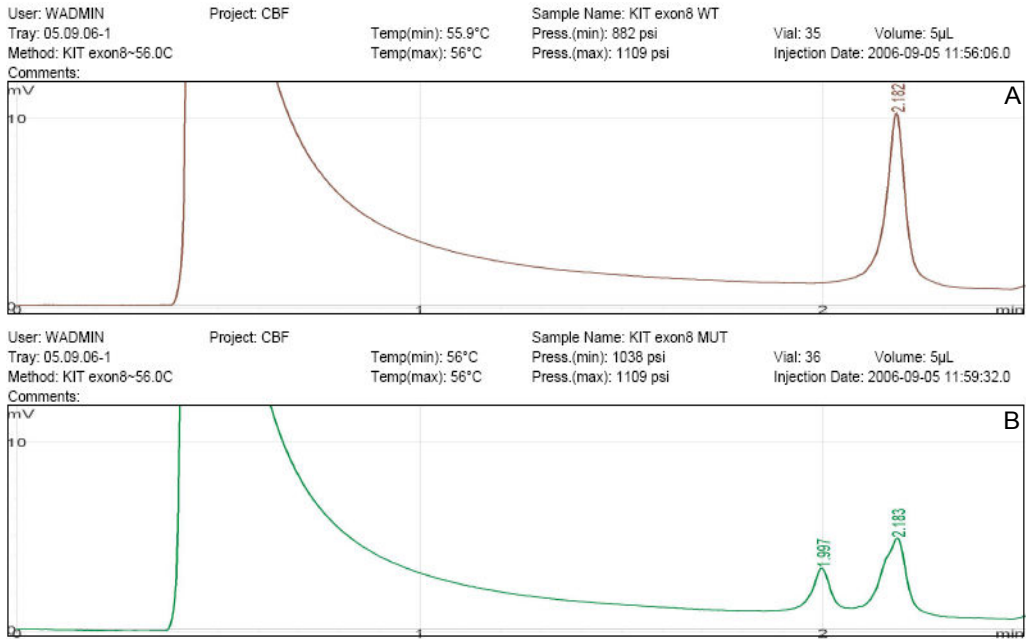

Figure 7. DHPLC profiles from representative *KIT* exon 8. A: DHPLC profiles from DNA containing *KIT* exon 8 wild-type; B: DNA containing *KIT* exon 8 mutant indicated by an additional peak. In the profiles indicating mutations, wild-type homoduplexes are retained on the DHPLC column for longer than wild-type/mutant heteroduplexes (*KIT* exon 8: 2.182 min, 1.997 min). DHPLC: Denaturing high performance liquid chromatography.

2.5.8. Purification of PCR products

For purification of DNA fragments, the QIAquick PCR Purification kit was used. Briefly, 25 µL of PCR product and 125 µL Buffer PBI were added to a QIAquick spin column for DNA binding and then centrifuged at 13,000 rpm for 1 min.. Discarded the flow-through, and then 750 µL washing Buffer PE was added to the QIAquick spin column for centrifuging at 13,000 rpm for 1 min.. After discarding flow-through, the column was centrifuged at 13,000 rpm for 1 min. again, and then the column was placed in a clean 1.5 mL microcentrifuge tube. Finally, 30 µL Buffer EB was

added to the center of QIAquick membrane for eluting. After incubation at room temperature for 1 min., the column was centrifuged again at 13,000 rpm for 1 min. to elute DNA. The purified PCR products were stored at -20°C until further used.

2.5.9. Cycle sequencing reaction (CSR)

Cycle sequencing reaction was performed using an *ABI* PRISM Big Dye Terminator Cycle Sequencing Kit, which contains thermally stable AmpliTaq DNA polymerase, modified dNTPs and a set of dye terminators labeled with high-senility dyes. The reaction was carried out in a total volume of 20 μL, containing 2 μL of purified PCR product, 1 μL (10 pmol/μL) of sequencing primer and 4 μL of Big Dye sequencing mix, using the following PCR profile: 25 cycles for 15 sec. at 96°C, 10 sec. at 54°C, and 4 min. at 60°C.

2.5.10. Dye removal using DyeEx spin kit

DyeEx 2.0 Spin Kit was used to remove unincorporated dye terminators directly from sequencing reactions. Firstly, the column was placed in a 2 mL collection tube and centrifuged 3 min. at 3.000 rpm to resuspend the resin. Secondly, the CSR product was loaded to gel bed and centrifuged for 3 min. at 3.000 rpm in a new collection tube. Finally, the purified DNA was transferred into sequencing tube. The purified PCR product was stored at -20°C until using for nucleotide sequencing.

2.5.11. Direct DNA sequencing

After denaturation at 95°C for 2 min., the samples were loaded on the *ABI*-PRISM310 genetic analyzer.

2.5.12. Sensitivity of DHPLC and sequencing assay

DNA isolated from a patient, who had 90% blast cells had one of targets mutation, was serially diluted by mixing with wide-type cells to create 10 different mixtures containing 90%, 75%, 50%, 25%, 15%, 10%, 5%, 4%, 2.5%, 1% positive DNAs, and then analyzed by DHPLC and sequencing as described above. When samples escaped sequencing, DHPLC in-line fragment collector was utilized as descried above.

2.6. Statistical analyses

The median duration of follow-up was calculated according to the method of Korn et al. (1986). The definition of CR followed the recommended criteria (Cheson et al. 2003). OS end points, measured from entry into one of the prospective studies, were death (failure) and alive at last follow-up (censored) (Cheson et al. 2003). RFS end points, measured from the date of documented CR, were relapse (failure), death in CR (failure), and alive in CR at last follow-up (censored) (Cheson et al. 2003). Event-free survival (EFS) was defined as the time from diagnosis to an event (either failure to achieve remission, death in first CR, or relapse). Pairwise comparisons between patient characteristics were performed by Mann-Whitney test for continuous variables and by Fisher exact test for categorical variables. A multivariable logistic model was used to analyze associations between presenting features and response to induction therapy. The Kaplan-Meier method was used to estimate the distribution of RFS, EFS and OS. Confidence interval (CI) estimation for the survival curves was based on the cumulative hazard function using Greenwood's formula for the standard error estimation (Therneau et al. 2000). Survival distributions were compared using the log-rank test. Missing data were estimated using a multiple-imputation technique using predictive mean matching with n = 100 for multiple imputations (Harrell et al. 2001). A limited backward selection procedure was used to exclude redundant or unnecessary variables (Harrell et al. 2001). To provide quantitative information on the relevance of results, 95% confidence intervals (95% CIs) of odds ratios (ORs) and hazard ratios (HRs) were computed. To correct for overestimation, a shrinkage procedure and a bootstrap correction of the CIs were applied according to Hollander et al. (2004). CIs for binomial probabilities were estimated using the method proposed by Agresti and Coull (1998). All statistical analyses were performed with R software, version 2.1.1, using R packages Design, version 2.0, and cmprsk, version 2.1 (all available at http://www.r-project.org) (R Development Core Team 2005).

3. Results

3.1. Incidence of *RAS*, *KIT*, *FLT3* and *JAK2* gene mutations

Mutations were identified in 87% of 101 inv(16) AML with highest frequencies in *NRAS* (51%, n = 51), followed by *KIT* (28%, n = 28) and *FLT3*-TKD (16%, n = 16); *KRAS* and *FLT3*-ITD mutations were detected in 9% and 4% of the patients, respectively (Figure 8). There were no differences in frequency of the distinct mutation types with respect to age (younger patients < 60 years versus older patients ≥ 60 years).

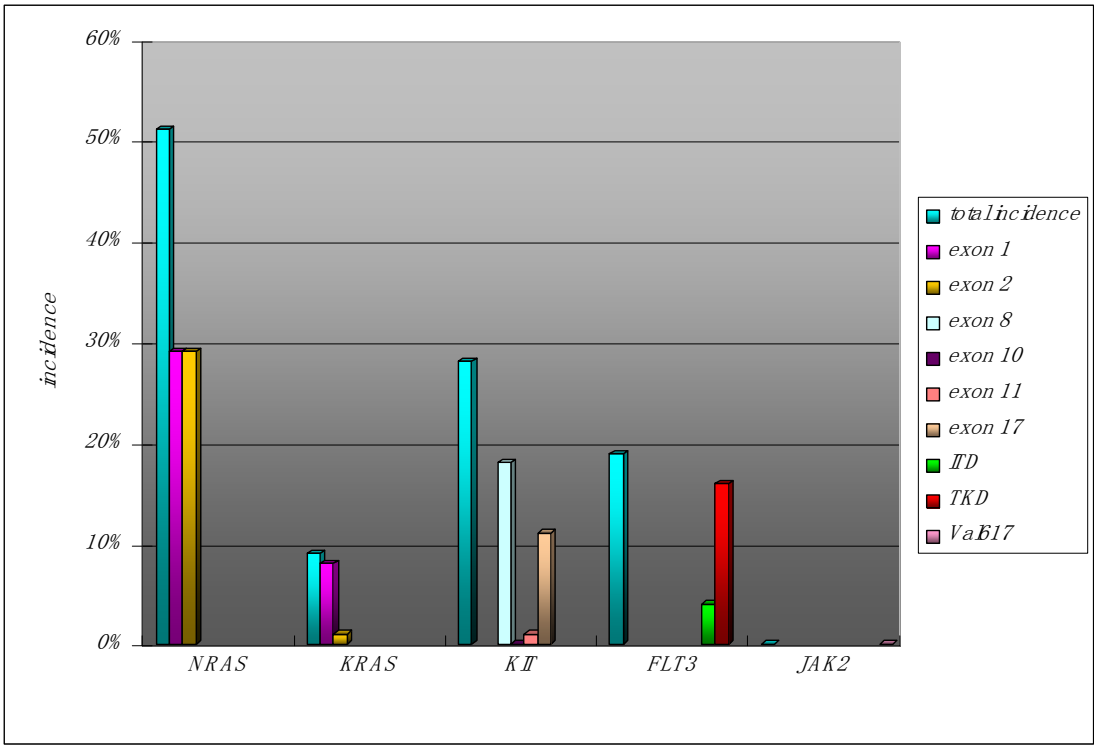

Figure 8. Distribution and incidence of *RAS*, *KIT*, *FLT3* and *JAK2* mutations in 101 inv(16)-positive patients.

In 66 t(8;21)-positive leukemias, mutations were found in 58% of patients with highest frequencies in *KIT* (24%, n = 16), followed by *NRAS* (21%, n = 14), *FLT3* (14%, n = 9), *JAK2* (6%, n = 4), and *KRAS* (5%, n = 3) (Figure 9).

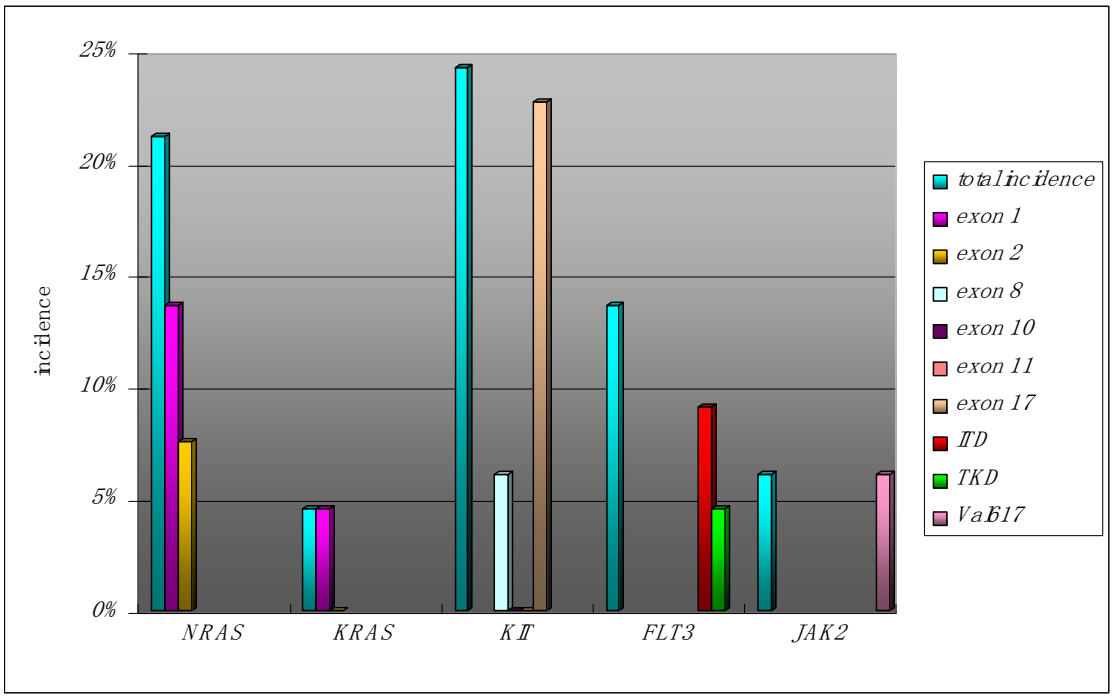

Figure 9. Distribution and incidence of *RAS*, *KIT, FLT3 and JAK2* mutations in 66 t(8;21)-positive patients.

3.1.1. *RAS* mutations

3.1.1.1. *RAS* mutations in inv(16) AML

Heterozygous *RAS* mutations were identified in 57% of the whole inv(16) group. The most frequent mutation type affected codon 61 (29%) of the *NRAS* gene, followed by mutations of codon 12 in 20%, and codon 13 in 9% of the patients. *KRAS* mutations were observed in 8% of all inv(16)-positive AML. In all of the cases mutations affected codon 12 and codon 13 located in exon 1 of *KRAS*. Sequence analysis identified two different exon 1 mutation types: A11_G12insA and G10dup. *KRAS* exon 2 mutation was only detected in one patient. A detailed distribution of mutations detected in *NRAS* and *KRAS* is shown in Figure 8.

3.1.1.2. *RAS* mutations in t(8;21) AML

Of the 66 t(8;21)-positive patients, *RAS* mutations were identified in 23% (n = 15) mostly affecting the *NRAS* gene (14 of 15 patients). Six of the 14 *NRAS* mutations (43%) occurred in codon 13, 5 (36%) in codon 61 and 3 (21%) in codon 12. Only 3 patients (2 patients coexisted *NRAS* mutation) exhibited *KRAS* exon 1 mutation and none of the patients had *KRAS* exon 2 mutations (Figure 9).

3.1.2. *KIT* mutations

3.1.2.1. *KIT* mutations in inv(16) AML

The incidence of *KIT* mutations in inv(16)-positive AML was 28% (n = 28) (Figure 8). In 28 mutated patients, 18 cases affected exon 8 mutation (18%), followed by exon 17 (11%, n = 10) and exon 11 mutations (1%, n = 1); among them, two patients harbored exon 8 as well as exon 17 mutations. None of samples had a *KIT* exon 10 mutation.

3.1.2.2. *KIT* mutations in t(8;21) AML

KIT mutation was identified in 24% (16/66) of the t(8;21)-positive patients. *KIT* exon 17 was detected in 15 (22%) patients, followed by 4 (6%) patients having *KIT* exon 8 mutations, among them, there were 3 patients who had both exon 8 and exon 17 mutations. In none of the patients *KIT* exons 10 or 11 mutations were identified (Figure 9).

3.1.2.3. Type of *KIT* mutations in inv(16) and t(8;21) AML

KIT exon 8 mutations were characterized as in-frame deletions or insertions involving codon 419. All *KIT* exon 17 mutations involved residues in the *KIT* activation loop which have recently been shown to result in constitutive kinase activity. The most commonly affected residue was codon 816, whereas less commonly mutations resulted in replacement of amino acids N822 and Y823.

In addition, three types of polymorphisms were identified in *KIT* exon 10 and exon 17: 1642 A>C, 1659 A>G, and 2415 C>T. *KIT* exon 10 1642 A>C was detected in 29 of 100 (29%) inv(16)-positive patients and in 12 of 66 (18%) t(8;21)-positive patients, respectively. Although 1642 A>C resulted in the substitution of a leucine for a methionine residue at codon 541, this type of mutation was also identified in remission samples of 10 of the affected patients suggesting that it is rather a polymorphism than an active mutation. These findings were supported by Nagata et al. (1996) who identified that sequence variation in a two-generation family as well as in a study by Fritsche-Polanz et al. (2001) where the 1642 A>C mutation was not more prevalent among patients than among healthy controls. The remaining two other polymorphisms were silent nucleotide

exchanges (*KIT* 1659 A>G in exon 10; *KIT* 2415 C>T in exon 17) not affecting the predicted amino acid sequence. *KIT* 1659 A>G mutation and *KIT* 2415 C>T mutations were identified in 7 (7%) and 4 (4%) of the inv(16) -positive patients, and in 3 (5%) and 5 (8%) of the t(8;21)-positive patients, respectively.

3.1.3. *FLT3* mutations

3.1.3.1. *FLT3*-ITD mutations in inv(16) and t(8;21) AML

FLT3-ITD mutations were identified in 4 (4%) of 101 inv(16)-, and 6 (9%) t(8;21)-positive AML patients, respectively (Figure 8, 9). In 6 of the 10 *FLT3*-ITD positive patients sequencing analysis could be performed showing a great variety of *FLT3*-ITDs in length ranging from 20 to 61 bp (median, 46). Five patients had simple *FLT3*-ITDs within exon 14, and in one patient *FLT3*-ITD involved exon 14 and intron 14. All *FLT3*-ITDs mutations were in-frame.

3.1.3.2. *FLT3*-TKD mutations in inv(16) and t(8;21) AML

Activating mutations affecting the second tyrosine kinase domain of the *FLT3* gene, the so called *FLT3*-TKD mutations, were detected in 16 (16%) of 101 inv(16) patients, and in 3 (5%) patients within t(8;21) cohort (Figure 8, 9). Most were point mutations resulting in a substitution of aspartic residue 835 with a tyrosine (D835Y, the most frequent mutation).

3.1.4. *JAK2* mutations

Heterozygous *JAK2* V617F mutations were identified in 4 (6%) t(8;21)-positive AML patients, whereas none of the inv(16)-positive cases showed sequence variations at codon 617 of the *JAK2* gene (Figure 8, 9).

3.2. Associated gene mutations

3.2.1. Associated gene mutations in inv(16)-positive patients

In this study patients were analyzed for mutations in the *NRAS*, *KRAS*, *KIT*, *FLT3*, and *JAK2* genes. 66 had only one single gene mutation whereas 21 patients exhibited more than one mutation either affecting the same gene or affecting one of the other genes mentioned above. Mostly the *NRAS/FLT3*-TKD genotype was

observed (n = 6), but also other genotypes such as *NRAS/KIT* (n = 5), *KIT*/*FLT3*-TKD (n = 3), *NRAS/KRAS* (n = 3), *KRAS/KIT* (n = 2), *FLT3*-ITD/ *FLT3*-TKD (n= 1) or *NRAS/FLT3*-ITD (n = 1) have been observed. A detailed distribution of the associated gene mutations is shown in Figure 10. Thus, 87 (86%) of 101 patients harbored at least one activating mutation.

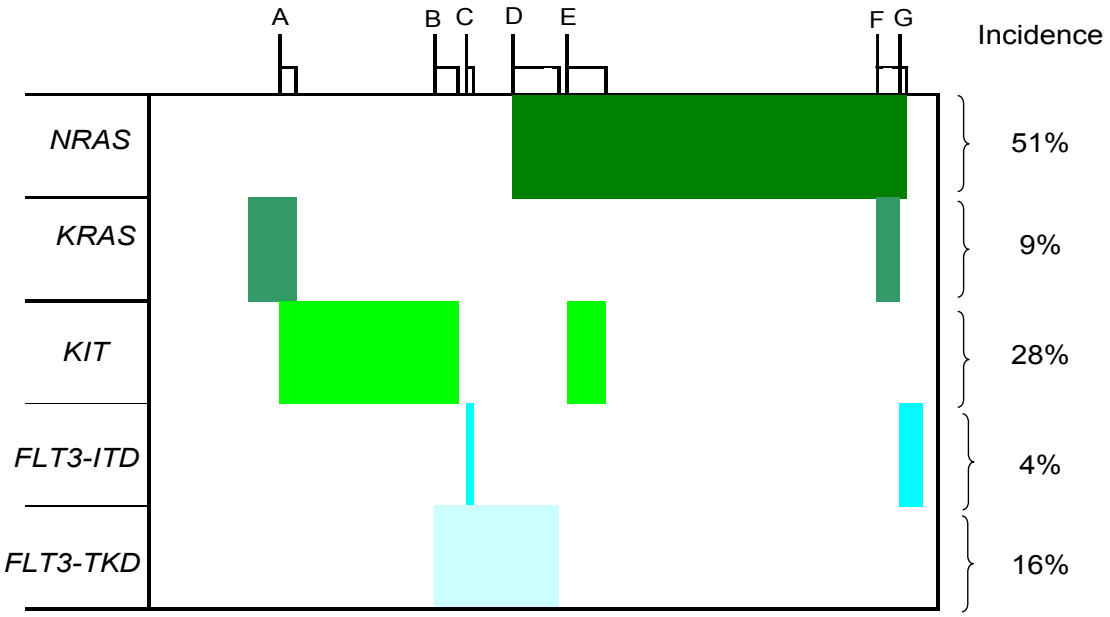

Figure 10. Associated gene mutation status based on 101 inv(16) patients for *NRAS, KRAS*, *KIT, FLT3*-ITD, and *FLT3*-TKD. A: *KRAS/KIT* n = 2; B: *KIT/FLT3-TKD* n = 3; C: *FLT3*-ITD/*FLT3*-TKD n = 1; D: *NRAS*/*FLT3*-TKD n = 6; E: *NRAS*/*KIT* n = 5; F: *NRAS*/*FLT3*-ITD n = 1; G: *NRAS*/*KRAS* n = 3.

3.2.2. Associated gene mutations in t(8;21)-positive patients

In the whole t(8;21)-positive group, 8 patients harbored two different gene mutations: 2 patients had the *NRAS*/*KRAS* genotype, 2 patients the *NRAS*/*KIT* genotype, two patient showed a combined *KIT*/*FLT3*-TKD mutation, and two patients with *JAK2* mutations revealed additional gain of function mutations in the genes encoding for *FLT3* and *NRAS*, respectively. A detailed distribution of the associated gene mutations is shown in Figure 11. Therefore, 58% patients presented at least one activating mutation.

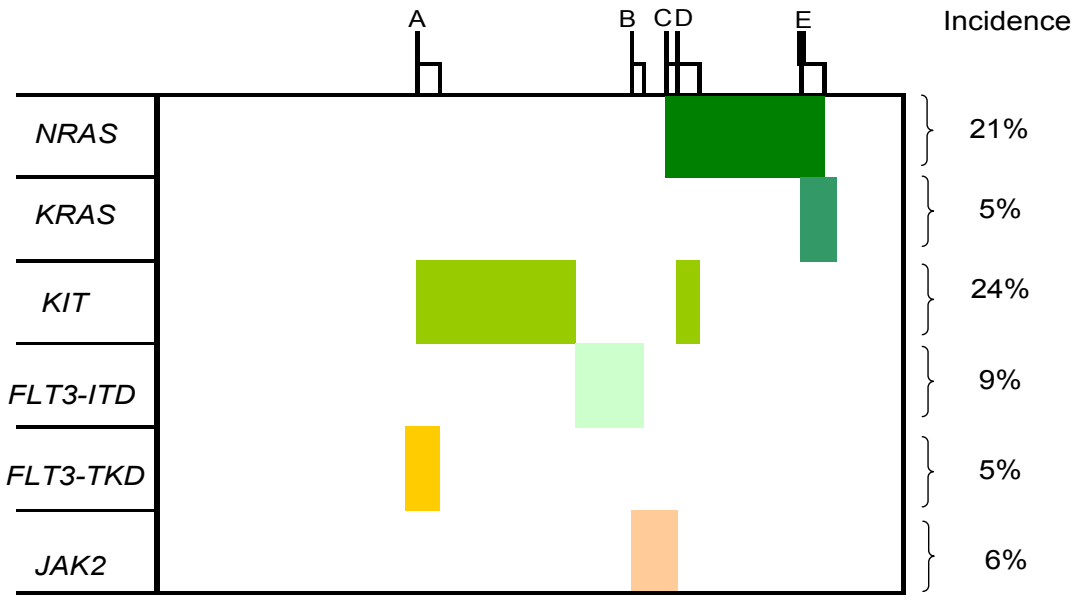

Figure 11. Associated gene mutation status based on 66 t(8;21) patients for *NRAS*, *KRAS*, *KIT*, *FLT3*-ITD, and *FLT3*-TKD. A: *KIT/FLT3*-TKD n = 2; B: *FLT3*-ITD/*JAK2* n = 1; C: *NRAS*/*JAK2* n = 1; D: *NRAS/KIT* n = 2; E: *NRAS*/*KRAS* n = 2.

3.3. Patients characteristics

3.3.1. Pretreatment characteristics for inv(16)-positive patients

Pretreatment characteristics of all inv(16) patients for *NRAS* and *KRAS* mutated patients are summarized in Table 6, and patients characteristics according to the *KIT* mutation status are shown in Table 7. Due to previous reports showing that *FLT3*-ITD and *FLT3*-TKD mutations show great differences in phenotype and clinical outcome, we additionally analyzed patients characteristics for inv(16)-positive AML according to the *FLT3*-ITD or *FLT3*-TKD mutation status (Table 8).

For the whole inv(16) group, the median age was 41 years (range 18 - 71 years), with 94 patients younger than 60 years of age (range 18 - 58 years). The majority (92%) had de novo AML, with FAB-M4 (67%) predominating. Correlation of the mutation status of each of the genes with clinical characteristics revealed only a few significant differences: patients with *KIT* mutation had a higher median percentage of PB blasts (62.5%) compared to unmutated patients (37%, $P = 0.004$). In addition to the aforementioned characteristics, subset analyses considering *KIT* exon 17 mutations revealed that mutated patients were more frequently male (P =

0.02). For *FLT3*-TKD mutated patients the analysis demonstrated that lactate dehydrogenase (LDH) serum levels in mutated patients were significantly higher (*P* = 0.04) compared to *FLT3*-TKD wild-type patients. Patients harboring *KIT* exon 17 mutations had in trend more frequently EML due to splenomegaly (*P* = 0.09), and this was also the case for *FLT3*-TKD positive patients (*P* = 0.09). There was no difference in WBC count between *RAS* mutated and unmutated patients. It is noteworthy that a lower percentage of PB blasts (36%) was found in *NRAS* mutated patients compared to unmutated patients (47%, *P* = 0.08); for *KRAS* a lower percentage of BM blasts was identified in mutated *KRAS* (51%) than in unmutated (80%) *KRAS* patients (*P* = 0.06). In addition, there was no significant difference for other presenting clinical features such as age, hemoglobin level, platelet count, or secondary chromosomal aberrations (e.g. trisomy 22) between patients with and without mutation in the genes *NRAS*, *KRAS*, *KIT* and *FLT3* (Table 6, 7, 8).

Within the younger inv(16) cohort, similar to the whole inv(16) groups, patients with *KIT* exon 17 mutations had significantly higher percentages of PB blasts (71.5%) compared to patients with *KIT* exon 17 wild-type (43.5%, *P* = 0.03), and showed to be in trend more frequently of male gender *(P* = 0.08). Since there was no statistical difference for the other clinical characteristic between patients with and without *NRAS*, *KRAS*, *FLT3* and *KIT* gene mutations in the whole inv(16) patients, subgroup analysis was not performed for the younger inv(16) group.

Table 6. inv(16)-positive AML: Patients characteristics according to the *NRAS* and *KRAS* mutation status.

Characteristics	*NRAS*-wt n = 49	*NRAS*-mut n = 51	*P* =	*KRAS*-wt n = 91	*KRAS*-mut n = 9	*P* =
Age, years Median (range)	40.58 (18.00-67.18)	41.56 (19.42-71.24)	0.94	41.03 (18.0-71.24)	41.82 (18.64-53.44)	0.49
<= 60 years s no.	45	48	0.71	84	9	1,00
>60 years no.	4	3		7	0	
Sex, no. of patients (%)						
Female	21	32	0.07	48	5	1,00
Male	28	19		43	4	
Type AML, no. of patients (%)						
De novo	43	49	0.12	83	9	1,00
s-AML	1	0		1	0	
t-AML	4	1		5	0	
missing	1	1		2		
Hemoglobin level,g/L, Median (range)	9.20 (2.50-14.20)	8.6 (3.0-12.5)	0.19	9.05 (2.5-14.2)	8.9 (7.4-11.1)	0.97
Platelet count,×10^9/L, Median (range)	35.0 (8.0-529.0)	33.5 (7.0-267.0)	0.73	34.5 (7.0-267.0)	30 (13-529)	0.37
WBC count,×10^9/L, Median (range)	35.8 (2.20-170.24)	36.65 (1.57-170.0)	0.99	36.65 (1.57-170.0)	31.50 (8.40-73.10)	0.60
PB blasts%, Median (range)	47 (0-97.0)	36.0 (5.0-91.0)	0.08	43.5 (0.0-97.0)	44.0 (12.0-71.0)	0.50
BM blasts% , Median (range)	80 (10.0-98.0)	79.00 (25.00-91.00)	0.44	80 (10.0-98.0)	51.0 (20-95.0)	0.07
FAB subtype, no. of patients (%)						
M0	1	0	0.62	1	0	0.71
M1	1	4		4	1	
M2	2	2		4	0	
M4	34	33		60	7	
M5	3	4		7	0	
M6	0	0		0	0	
missing	8	8		15	1	
Trisomy 22, no. of patients (%)	10(20)	8(16)	0.75	17(20)	1(13)	0.61
missing no.	2	3		4	1	
LDH, U/L, Median (range)	546.0 (150-2570.0)	568 (200-1864)	0.99	573.0 (150-2570.0)	461.0 (317-1467)	0.38
Splenomegaly,*no. (%)	30(71)	22(56)	0.20	50(66)	2(40)	0.06
missing no.	7	12		15	4	
Hepatomegaly, no. (%)	13(39)	13(43)	0.63	23(40)	3(50)	0.82
missing no.	16	21		34	3	

AML: acute myeloid leukemia; BM: bone marrow; FAB: French-American-British; LDH: lactate dehydrogenase, normal value less than 240 U/L; PB: Peripheral Blood; t-AML: therapy-related AML; s-AML: secondary AML; -mut: Mutation; WBC: white blood cell; -wt: -Wild-type. *Defined as more than 13 cm as measured by ultrasonography.

Table 7. inv(16)-positive AML: Patients characteristics according to the *KIT* mutation status.

Characteristics	*KIT*-wt n = 72	*KIT*-mut n = 28	*P* =	*KIT*17-wt n = 89	*KIT*17-mut n = 11	*P* =
Age, years Median (range)	42.25 (18.96-71.24)	37.33 (18.00-68.05)	0.20	41.03 (18.00-71.24)	43.74 (22.93-67.18)	0.94
<= 60 years s no.	67	26	1,00	83	10	0.57
>60 years no.	5	2		6	1	
Sex, no. of patients (%)						
Female	42	11	0.12	51	2	0.02
Male	30	17		38	9	
Type AML, no. of patients (%)						
De novo	66	26	0.39	82	10	0.12
s-AML	0	1		0	1	
t-AML	4	1		5	0	
missing	2			2		
Hemoglobin level,g/L, Median (range)	8.80 (5.2-12.7)	9.20 (2.50-14.20)	0.51	9.05 (3.00-14.2)	8.60 (2.50-11.90)	0.94
Platelet count,×10^9/L, Median (range)	34.0 (7.0-267.0)	34.0 (15.0-529.0)	1,00	34.0 (7.0-529.0)	34.0 (17.0-110.0)	0.86
WBC count,×10^9/L, Median (range)	34.80 (1.57-170.24)	49.00 (2.40-165.60)	0.14	35.90 (1.57-170.24)	42.90 92.40-121.70)	0.98
PB blasts%, Median (range)	37.00(0.0-91.00)	62.50 (13.00-97.00)	0.004	38.5 (0.0-91.0)	67.0 (36.0-97.0)	0.007
BM blasts% , Median (range)	80.0 (20.0-97.0)	75.00 910.0-98.0)	0.74	79.5 (20.0-98.0)	77.5 (10.0-90.0)	0.98
FAB subtype, no. of patients (%)						
M0	0	1	0.20	1	0	0.63
M1	4	1		5	0	
M2	3	1		3	1	
M4	47	20		57	10	
M5	7	0		7	0	
M6	0	0		0	0	
missing	11	5		16		
Trisomy 22, no. of patients (%)	13(19)	5(19)	0.76	16(19)	2(20)	0.57
missing no.	3	2		4	1	
LDH, U/L, Median (range)	568.0 (150.0-2570.0)	563.0 (200.0-1164.0)	0.56	566 (150-2570)	511.0 (298-1164)	0.72
Splenomegaly,*no. (%)	34(61)	18(72)	0.29	43(61)	9(82)	0.09
missing no.	16	3		19		
Hepatomegaly, no. (%)	17(40)	9(45)	0.53	20(64)	6(75)	0.12
missing no.	29	8		34	3	

AML: acute myeloid leukemia; BM: bone marrow; FAB: French-American-British; LDH: lactate dehydrogenase, normal value less than 240 U/L; PB: Peripheral Blood; t-AML: therapy-related AML; s-AML: secondary AML; -mut: Mutation; WBC: white blood cell; -wt: -Wild-type. *Defined as more than 13 cm as measured by ultrasonography.

Table 8. inv(16)-positive AML: Patients characteristics according to the *FLT3* mutation status.

Characteristics	*FLT3*-ITD-wt n = 97	*FLT3*-ITD-mut n = 4	*P* =	*FLT3*-TKD-wt n = 85	*FLT3*-TKD-mut n = 16	*P* =
Age, years Median (range)	41.03 (18.00-71.24)	46.78 (37.16-66.52)	0.25	43.17 (18.00-71.24)	37.71 (22.93-51.52)	0.14
<= 60 years s no.	91	3	0.25	78	16	0.59
>60 years no.	6	1		7	0	
Sex, no. of patients (%)						
Female	53	1	0.34	46	8	0.79
Male	44	3		39	8	
Type AML, no. of patients (%)						
De novo	90	3	1,00	79	14	0.12
s-AML	1	0		0	1	
t-AML	5	0		4	1	
missing	1	1		2		
Hemoglobin level,g/L, Median (range)	8.90 (2.50-14.20)	10.60 (6.90-12.50)	0.42	9.15 (3.0-14.20)	8.50 (2.50-12.70)	0.18
Platelet count,×10^9/L, Median (range)	34.0 (7.0-529.0)	52.0 (46.0-82.0)	0.18	37.5 (8.0-529.0)	30.5 (7.0-96.0)	0.19
WBC count,×10^9/L, Median (range)	37.30 (1.57-170.24)	35.80 (7.6-43.4)	0.49	33.65 (1.57-165.60)	67.00 (2.68-170.24)	0.98
PB blasts%, Median (range)	45.0 (0.0-97.0)	37.0 (11.0-42.0)	0.33	41.0 (0.0-97.0)	50.0 (7.0-90.0)	0.59
BM blasts%, Median (range)	80.0 (10.0-98.0)	77.0 (30.0-80.0)	0.39	79.0 (10.0-98.0)	81.5 (20.0-91.0)	0.89
FAB subtype, no. of patients (%)						
M0	1	0	0.49	1	0	0.60
M1	5	0		5	0	
M2	4	0		3	1	
M4	66	2		57	11	
M5	6	1		5	2	
M6	0	0		0	0	
missing	16	1		14	3	
Trisomy 22, no. of patients (%)	18(20)	0	1,00	13(16)	5(31)	0.31
missing no.	5			5		
LDH, U/L, Median (range)	564.5 (150.0-2570.0)	492.0 (459.0-581.0)	0.66	522 (150.0-1864)	641.0 (296.0-2570.0)	0.04
Splenomegaly,*no. (%)	49(63)	3(100)	0.52	41(59)	11(92)	0.09
missing no.	19	1		16	4	
Hepatomegaly, no. (%)	26(42)	1(50)	1,00	23(42)	4(44)	0.84
missing no.	35	2		30	7	

AML: acute myeloid leukemia; BM: bone marrow; FAB: French-American-British; LDH: lactate dehydrogenase, normal value less than 240 U/L; PB: Peripheral Blood; t-AML: therapy-related AML; s-AML: secondary AML; -mut: Mutation; WBC: white blood cell; -wt: -Wild-type. *Defined as more than 13 cm as measured by ultrasonography.

3.3.2. Pretreatment characteristics for t(8;21)-positive patients

The pretreatment characteristics of the 66 t(8;21)-positive AML patients according to the *NRAS* and *KRAS*, *KIT* (including exon 17) and *FLT3* (ITD and TKD) mutation status are given in Table 9, 10 and 11, respectively. Owing to the incidence of J*AK2* mutations was less (n = 4), statistical correlation did not reveal any significant features when comparing mutated and unmutated t(8;21)-patients. The median age was 49 years in the whole group (range 18 - 73 years), with 52 patients younger than 60 years of age (range 18 - 59). In 71% patients had de novo AML and the most common FAB subtype was M2 (60%) in all t(8;21) patients.

Subgroup analysis for the other genotypes showed a significant higher LDH serum levels for *KIT*-mutated patients (*P* = 0.004). The same was true when subgroup analysis for *KIT* exon 17 mutation was performed (*P* = 0.004). The higher frequency of EML involvement in the *KIT* exon 17 mutated group was due to higher rate of hepatomegaly (*P* = 0.04). In *NRAS* mutated patients a significantly lower median percentage BM blast (50%) was found compared to unmutated patients (70%, *P* = 0.04); in analogy, *KRAS* mutations had significantly lower BM blast (32%) than patients with *KRAS* wild-type (60%, *P* = 0.05). Secondary chromosome aberrations, such as loss of Y chromosome in male patients which was observed in 51% of the patients (21/41) or loss of X chromosome in female patients that was found in (36%) of the patients (9/25) no statistically significant differences between mutated and unmutated patients was observed with regard to *RAS*, *KIT*, *FLT3*, or *JAK2* genes. In addition, correlation of other clinical characteristics such as age, hemoglobin level, platelet count, BM blast, or other EML did not reveal significant differences between the mutated and unmutated patient subgroups (Table 9, 10, 11).

We further correlated the patient characteristics for the younger t(8;21)-positive patients group. In analogy to the whole group, the main difference was observed in patients with *KIT*-mutations showing a significantly higher LDH than *KIT*-unmutated patients (*P* = 0.007), this difference was also significant when focusing on *KIT* exon 17 mutations (*P* = 0.007). The *KIT* exon 17 mutated group also had a higher rate of hepatomegaly (*P* = 0.05). In contrast to the whole t(8;21) group, there was no difference in the percentages of PB and BM blasts for the

younger group with regard to *NRAS* and *KRAS* mutation status. Secondary chromosome abnormality such as loss of Y chromosome was observed in 48% of the younger patients (15/31), loss of X chromosome in female patients occurred in 38% (8/21) of the younger patients. There was no difference between mutated and unmutated patients with regard to the *RAS*, *KIT* or *FLT3* mutation status. In addition, correlation of other clinical characteristics such as age, hemoglobin level, platelet count, BM blast, or other EML did not reveal significant differences between the mutated and unmutated groups in younger patients.

Table 9. t(8;21)-positive AML: Patients characteristics according to the *NRAS* and *KRAS* mutation status.

Characteristics	*NRAS*-wt n = 52	*NRAS*-mut n = 14	*P* =	*KRAS*-wt n = 63	*KRAS*-mut n= 3	*P* =
Age, years Median (range)	49.19 (18.440-73.09)	50.21 (19.16-63.52)	0.83	49.11 (18.44-73.09)	50.59 (45.01-63.52)	0.64
<= 60 years no.	40	12		50	2	
>60 years no.	12	2		13	1	
Sex, no. of patients (%)						
Female	19	6	0.76	25	0	0.28
Male	33	8		38	3	
Type AML, no. of patients (%)						
De novo	38	9	0.41	46	1	0.09
s-AML	3	0		6	2	
t-AML	5	3		3	0	
missing	6	2		9		
Hemoglobin level, g/L, Median (range)	9.10 (4.20-14.80)	8.0 (4.7-11.2)	0.32	9.0 (4.2-14.8)	8.00 (5.40-9.70)	0.33
Platelet count,×10^9/L, Median (range)	36.2 (5.0-226.0)	45.0 (7.0-470.0)	0.12	39.0 (5.0-470.0)	42.0 (30.0-58.0)	0.83
WBC count,×10^9/L, Median (range)	9.2 (1.1-133.2)	17.00 (3.50-152.0)	0.07	22.16 (1.10-152.0)	17.00 (9.40-22.10)	0.41
PB blasts%, Median (range)	46.5 (1.0-90.0)	34.0 (0.0-87.0)	0.12	46.0 (0.0-90.0)	25.0 (10.0-31.0)	0.09
BM blasts%, Median (range)	70.0 (6.0-85.0)	50.0 (20.0-80.0)	0.04	60.0 (6.0-98.0)	32.0 (25.0-38.0)	0.05
FAB subtype, no. of patients (%)						
M0	2	1	0.85	3	0	0.17
M1	5	1		5	1	
M2	32	7		38	1	
M3	2	1		2	1	
M4	4	1		5	0	
M5	0	0		0	0	
M6	0	0		0	0	
missing	7	3		10		
Additional Chromosomal Aberrations, no. of patients (%)						
Loss Y	16(31)	5(42)	0.25	19(32)	2(67)	0.76
Loss X	8(16)	1(8)		9(15)	0	
missing no.	1	2		3		
LDH, U/L, Median (range)	470.0 (161.0-3890.0)	740.0 (366.0-1299.0)	0.10	497.0 (161.0-3890.0)	740.0 (375.0-1096.0)	0.48
Splenomegaly,*no. (%)	13(41)	4(40)	0.80	16(42)	1(33)	0.47
missing no.	20	4		24		
Hepatomegaly, no. (%)	6(27)	0	0.16	6(21)	0	1.00
missing no.	30	6		34	2	

AML: acute myeloid leukemia; BM: bone marrow; FAB: French-American-British; LDH: lactate dehydrogenase, normal value less than 240 U/L; PB: Peripheral Blood; t-AML: therapy-related AML; s-AML: secondary AML; -mut: Mutation; WBC: white blood cell; -wt: -Wild-type. *Defined as more than 13 cm as measured by ultrasonography.

Table 10. t(8;21)-positive AML: Patients characteristics according to the *KIT* mutation status.

Characteristics	*KIT*-wt n = 50	*KIT*-mut n = 16	*P* =	*KIT*17-wt n = 51	*KIT*17-mut n = 15	*P* =
Age, years Median (range)	49.76 (18.44-73.09)	46.63(19.16-72.37)	0.96	49.27 (18.440-73.09)	47.85 (33.87-72.37)	0.68
<= 60 years no.	41	11		42	10	
>60 years no.	9	5		9	5	
Sex, no. of patients (%)						
Female	19	6	1.00	19	6	1.00
Male	31	10		32	9	
Type AML, no. of patients (%)						
De novo	34	13	0.85	35	12	1.00
s-AML	6	2		6	2	
t-AML	3	0		3	0	
missing	7	1		7	1	
Hemoglobin level,g/L, Median (range)	8.8 (4.2-12.6)	9.40 (6.60-14.80)	0.36	8.9 (4.2-12.6)	9.55 (6.60-14.80)	0.40
Platelet count,×10^9/L, Median (range)	39.0 (7.0-470.0)	35.0 (5.0-114.0)	0.53	40.5 (7.0-470.0)	34.0 (5.0-114.0)	0.33
WBC count,×10^9/L, Median (range)	9.40 (1.10-152.00)	10.35 (2.30-60.45)	0.73	10.10 (1.10-152.00)	9.90 (2.3-36.30)	0.43
PB blasts%, Median (range)	46.0 (0.0-90.0)	50.0 (1.0-87.0)	0.43	46 (0-90)	47.5 (1.0-78.0)	0.76
BM blasts%, Median (range)	50.5 (6.0-98.0)	65.0 (24.0-93.0)	0.27	51.0 (6.0-98.0)	70.0 (24.0-93.0)	0.24
FAB subtype, no. of patients (%)						
M0	2	1	0.28	2	1	0.18
M1	5	1		5	1	
M2	31	8		32	7	
M3	2	1		2	1	
M4	2	3		2	3	
M5	0	0		0	0	
M6	0	0		0	0	
missing	8	2		8	2	
Additional Chromosomal Aberrations, no. of patients (%)						
Loss Y	17 (36)	4(25)	0.67	17(33)	4(27)	0.75
Loss X	6(13)	3(19)		6(13)	3(20)	
missing no.	3			3		
LDH, U/L, Median (range)	439.5 (161.0-3890.0)	895.0 (213.0-2579.0)	0.004	439.5 (161.0-3890.0)	895.5 (213.0-2579.0)	0.004
Splenomegaly,*no. (%)	13(36)	4(50)	0.37	14(39)	3(43)	0.35
missing no.	16	8		16	8	
Hepatomegaly, no. (%)	2(9)	4(50)	0.06	2(9)	4(50)	0.04
missing no.	28	8		29	7	

AML: acute myeloid leukemia; BM: bone marrow; FAB: French-American-British; LDH: lactate dehydrogenase, normal value less than 240 U/L; PB: Peripheral Blood; t-AML: therapy-related AML; s-AML: secondary AML; -mut: Mutation; WBC: white blood cell; -wt: -Wild-type. *Defined as more than 13 cm as measured by ultrasonography.

Table 11. t(8;21)-positive AML: Patients characteristics according to the *FLT3* mutation status.

Characteristics	*FLT3*- ITD-wt n = 60	*FLT3*- ITD-mut n = 6	*P* =	*FLT3*-TKD-wt n = 63	*FLT3*-TKD-mut n = 3	*P* =
Age, years Median (range)	49.75 (18.44-73.09)	44.99 (33.56-58.96)	0.27	49.11 (19.16-73.09)	68.810 (18.44-70.41)	0.56
<= 60 years no.	46	6		51	1	
>60 years no.	14	0		12	2	
Sex, no. of patients (%)						
Female	22	3	0.67	23	2	0.55
Male	28	3		40	1	
Type AML, no. of patients (%)						
De novo	42	5	0.31	46	1	1.00
s-AML	8	0		8	0	
t-AML	2	1		3	0	
missing	8			6	2	
Hemoglobin level,g/L, Median (range)	9.05 (4.70-14.80)	8.75 (4.20-9.80)	0.48	9.00 (4.20-14.80)	10.6 (10.6-10.6)	0.23
Platelet count,×10^9/L, Median (range)	37.1 (5.0-470.0)	52.0 (20.0-191.0)	0.20	39.0 (5.0-470.0)	9.0 (9.0-9.0)	0.13
WBC count,×10^9/L, Median (range)	9.9 (1.8-152.0)	11.15 (1.10-133.20)	0.54	9.9 (1.1-152.0)	9.8 (2.6-17.0)	0.62
PB blasts%, Median (range)	46.0 (0.0-90.0)	68.0 (24.0-79.0)	0.37	46.0 (0.0-90.0)	50.0 (50.0-50.0)	0.81
BM blasts%, Median (range)	60.0 (6.0-98.0)	50.5 (30.0-85.0)	0.66	60.0 (6.0-98.0)	93.0 (93.0-93.0)	0.17
FAB subtype, no. of patients (%)						
M0	3	0	1.00	3	0	1.00
M1	6	0		6	0	
M2	34	5		37	2	
M3	3	0		3	0	
M4	5	0		5	0	
M5	0	0		0	0	
M6	0	0		0	0	
missing	9	1		9	1	
Additional Chromosomal Aberrations, no. of patients (%)						
Loss Y	20(34)	1(20)	0.34	21(35)	0	0.36
Loss X	8(14)	1(20)		8(13)	1(33)	
missing no.	2	1		3		
LDH, U/L, Median, range	489.0 (161.0-2579.0)	804.5 (249.0-3890)	0.38	497.0 (161.0-3890.0)	1759.0 (1759.0-1759.0)	0.12
Splenomegaly,*no. (%)	15(42)	2(33)	0.12	17(40)	0	0.06
missing no.	24			21	3	
Hepatomegaly, no. (%)	6(22)	0	0.82	6(20)	0	0.45
missing no.	33	3		33	3	

AML: acute myeloid leukemia; BM: bone marrow; FAB: French-American-British; LDH: lactate dehydrogenase, normal value less than 240 U/L; PB: Peripheral Blood; t-AML: therapy-related AML; s-AML: secondary AML; -mut: Mutation; WBC: white blood cell; -wt: -Wild-type. *Defined as more than 13 cm as measured by ultrasonography.

3.4. Response to induction therapy

3.4.1. Response to induction therapy for inv(16)-positive patients

The CR rate in younger (< 60 years) and older (≥ 60 years) patients was 89% and 85%, respectively. Correlations between the presence of *NRAS*, *KRAS*, *KIT*, and *FLT3* mutations and response to induction therapy, as determined by univariate analysis, are given in Table 12, 13, and 14. Univariate analysis showed that the presence of *FLT3*-ITD mutations was associated with low CR (50%), 25% resistant disease (RD), and 25% early death (ED) ($P = 0.06$). However, the number of *FLT3*-ITD mutated patients was quite small (n = 4), 1 patient had RD, 1 had ED, and 2 patients attained CR. There was no difference in response to induction therapy with regard to the *NRAS*, *KRAS*, *KIT* and *FLT3*-TKD mutational status when tested as single variables (Table 12, 13, 14). Due to very low RD and ED, multivariable analysis could not be performed in response rates after induction therapy for the different gene mutations.

3.4.2. Response to induction therapy for t(8;21)-positive patients

For all 66 patients with t(8;21) the CR rate was 86%; whereas 90% of the younger patients achieved CR after induction therapy. Univariate analyses revealed that there was no difference in CR rates for *RAS, KIT, FLT3* (Table15, 16, 17), and *JAK2* (data not show) mutated t(8;21)-positive patients compared to the unmutated group. Due to very low RD and ED, multivariable analysis could not be performed.

Table 12. Achievement of CR according to the *NRAS* and *KRAS* mutation status in inv(16)-positive patients.

			NRAS-wt n = 49	*NRAS*-mut n = 51		*KRAS*-wt n = 91	*KRAS*-mut n = 9	
Remission	**All Patients n = 101**	**age < 60 years n = 94**	**no. of patients (%)**	**no. of patients (%)**	***P* =**	**no. of patients (%)**	**no. of patients (%)**	***P* =**
CR	90 (89)	85(90)	42(86)	47(92)	0.06	82(90)	7(78)	0.26
RD	5 (5)	3(3)	5(10)	0		4(4)	1(11)	
ED	6 (6)	6(7)	2(4)	4(8)		5(5)	1(11)	

-wt: -Wild-type; -mut: Mutation; CR indicated complete remission; RD: resistant disease; ED: early death.

Table 13. Achievement of CR according to the *KIT* mutation status in inv(16)-positive patients.

			KIT-wt n = 72	*KIT*-mut n = 28		*KIT*17-wt n = 89	*KIT*17-mut n = 11	
Remission	**All Patients n = 101**	**age < 60 years n= 94**	**no. of patients (%)**	**no. of patients (%)**	***P* =**	**no. of patients (%)**	**no. of patients (%)**	***P* =**
CR	90 (89)	85(90)	62(86)	27(96)	0.37	78(87)	11(100)	1,00
RD	5 (5)	3(3)	4(6)	1(4)		5(6)	0	
ED	6 (6)	6(7)	6(8)	0		6(7)	0	

-wt: -Wild-type; -mut: Mutation; CR indicated complete remission; RD: resistant disease; ED: early death.

Table 14. Achievement of CR according to the *FLT3* mutation status in inv(16)-positive patients.

			FLT3-TKD-wt n = 85	*FLT3*-TKD-mut n = 16		*FLT3*-ITD-wt n = 97	*FLT3*-ITD-mut n = 4	
Remission	**All Patients n=101**	**age < 60 years n= 94**	**no. of patients (%)**	**no. of patients (%)**	***P* =**	**no. of patients (%)**	**no. of patients (%)**	***P* =**
CR	90 (89)	85(90)	75(88)	15(94)	1,00	88(91)	2(50)	0.06
RD	5 (5)	3(3)	5(6)	0		4(4)	1(25)	
ED	6 (6)	6(7)	5(6)	1(6)		5(5)	1(25)	

-wt: -Wild-type; -mut: Mutation; CR indicated complete remission; RD: resistant disease; ED: early death.

Table 15. Achievement of CR according to the *NRAS* and *KRAS* mutation status in t(8;21)-positive patients.

			NRAS-wt n = 52	*NRAS*-mut n = 14		*KRAS*-wt n = 63	*KRAS*-mut n = 3	
Remission	**All Patients n = 66**	**age < 60 years n = 52**	**no. of patients (%)**	**no. of patients (%)**	***P* =**	**no. of patients (%)**	**no. of patients (%)**	***P* =**
CR	55 (86)	46(90)	44(86)	11(84)	0.45	53(87)	2(67)	0.43
RD	3 (5)	0	2(4)	1(8)		3(5)	0	
ED	6 (9)	5(10)	5(10)	1(8)		5(8)	1(33)	
missing			1	1		2		

-wt: -Wild-type; -mut: Mutation; CR indicated complete remission; RD, resistant disease; and ED, early death.

Table 16. Achievement of CR according to the *KIT* mutation status in t(8;21)-positive patients.

			KIT-wt n = 50	*KIT*-mut n = 16		*KIT*17-wt n = 51	*KIT*17-mut n = 15	
Remission	**All Patients n = 66**	**age < 60 years n = 52**	**no. of patients (%)**	**no. of patients (%)**	***P* =**	**no. of patients (%)**	**no. of patients (%)**	***P* =**
CR	55 (86)	46(90)	43(88)	12(80)	0.18	44(88)	11(79)	0.14
RD	3 (5)	0	1(2)	2(13)		1(2)	2(14)	
ED	6 (9)	5(10)	5(10)	1(7)		5(10)	1(7)	
missing			1	1		1	1	

-wt: -Wild-type; -mut: Mutation; CR indicateds complete remission; RD, resistant disease; and ED, early death.

Table 17. Achievement of CR according to the *FLT3* mutation status in t(8;21)-positive patients.

			FLT3 ITD-wt n = 60	*FLT3* ITD-mut n = 6		*FLT3*-TKD-wt n = 63	*FLT3*-TKD-mut n = 3	
Remission	**All Patients n = 66**	**age < 60 years n = 52**	**no. of patients (%)**	**no. of patients (%)**	***P* =**	**no. of patients (%)**	**no. of patients (%)**	***P* =**
CR	55 (86)	46(90)	50(86)	5(83)	0.68	53(85)	2(100)	0.14
RD	3 (5)	0	3(5)	0		3(5)	0	
ED	6 (9)	5(10)	5(9)	1(17)		6(10)	0	
missing			2			1	1	

-wt: -Wild-type; -mut: Mutation; CR indicateds complete remission; RD, resistant disease; and ED, early death.

3.5. Survival analysis

3.5.1. Survival analysis for inv(16)-positive patients

In the whole inv(16) group, 94 patients received the assigned postremission therapy. The median follow-up time for survival was 52 months (95%-CI, 36 - 62 months). In univariate analyses, *FLT3*-TKD mutations were associated with a significant inferior RFS (*P* = 0.03), and in trend adverse EFS (*P* = 0.07) (Figure 12), but not in OS (*P* = 0.55). Of the 4 *FLT3*-ITD-mutated patients, 2 died from disease progression during chemotherapy, and 2 are alive after autologous SCT, therefore, no significant difference in OS (*P* = 0.15) and RFS (*P* = 0.86) was observed. For the patients harboring *NRAS* mutations, there was no significant difference in OS (*P* = 0.52), EFS (*P* = 0.59), and RFS (*P* = 0.80). The same was true when comparing patients with and without *KRAS* mutations; again there was no significant difference in OS (*P* = 0.85), EFS (*P* = 0.99), and RFS (*P* = 0.62). However, the *NRAS* mutated group showed a trend to reach a plateau after 1 year of follow-up for RFS (*P* = 0.80) as well as EFS (*P* = 0.59) for (Figure 13). This feature was not observed in the *KRAS* mutated groups, which is probably due to the low number (n = 9) of mutated patients. Between *KIT*-mutated and *KIT*-unmutated patients there was no significant difference in OS (*P* = 0.70), EFS (*P* = 0.86), and RFS (*P* = 0.38); when focusing at *KIT* exon 17 mutation only, again there was no significant difference in OS (*P* = 0.66), EFS (*P* = 0.59), and RFS (*P* = 0.23) between the mutated and unmutated patients. However, when performing the analyses according to the different postremission therapies [high-dose cytarabine (HiDAC), autologous SCT (autoSCT), allogeneic SCT (alloSCT)], the distinct consolidation regimens seem to impact the prognosis for *FLT3*-TKD and *KIT* exon 17 mutated patients.

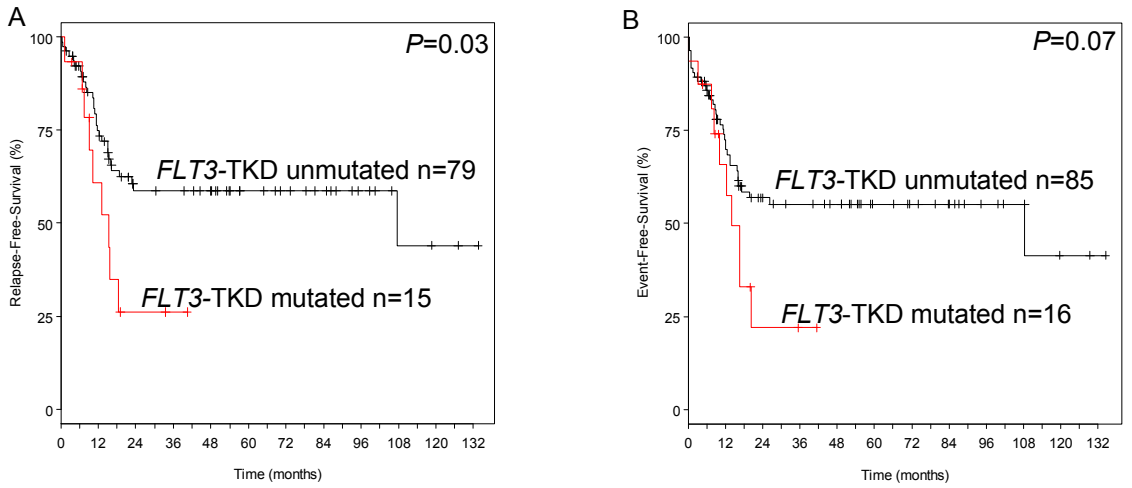

Figure 12. RFS (A) and EFS (B) according to the *FLT3*-TKD mutation status in 101 inv(16) patients. EFS: event-free survival; RFS: relapse-free survival.

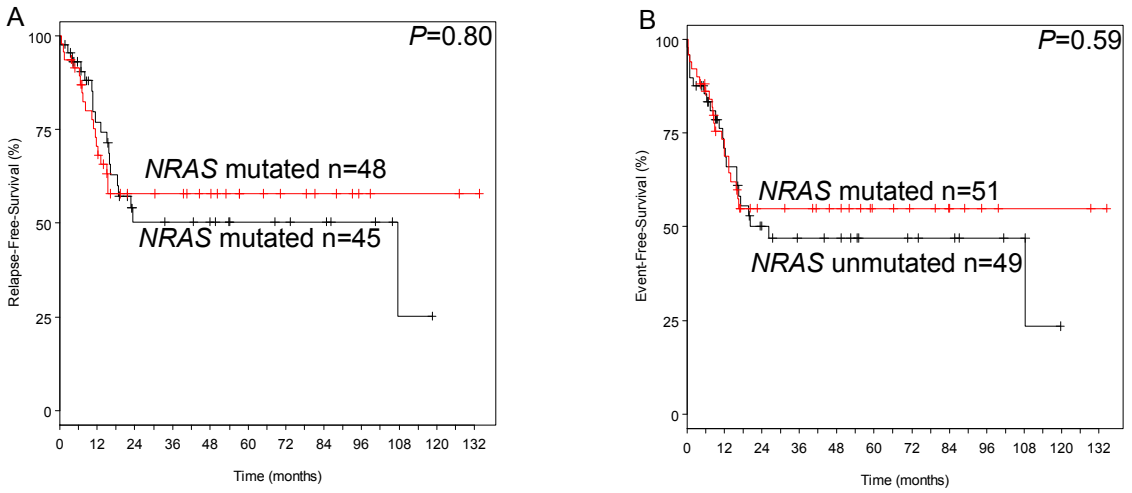

Figure 13. RFS (A) and EFS (B) according to the *NRAS* mutation status in 101 inv(16) patients. EFS: event-free survival; RFS: relapse-free survival.

Based on the different postremission therapies that patients with *FLT3*-TKD mutations received, we defined 5 subgroups for further analysis: alloSCT-*FLT3*-TKD-unmutated (n = 13), autoSCT-*FLT3*-TKD-unmutated (n = 18), autoSCT- *FLT3*-TKD mutated (n = 5), HiDAC- *FLT3*-TKD-unmutated (n = 47), HiDAC-*FLT3*-TKD mutated (n = 10); there was no *FLT3*-TKD-mutated patient who was assigned to alloSCT. The estimated 3-year RFS and EFS among these 5 groups was significantly different (P = 0.003 and P = 0.007, respectively) (Figure 14). *FLT3*-TKD mutated patients undergoing autoSCT had a better RFS than *FLT3*-TKD mutated patients receiving HiDAC, and unmutated patients treated with

more intensive regimens such as alloSCT or autoSCT, had a better RFS compared to patients treated with HiDAC alone. There was no significant difference in OS among these groups (*P* = 0.24).

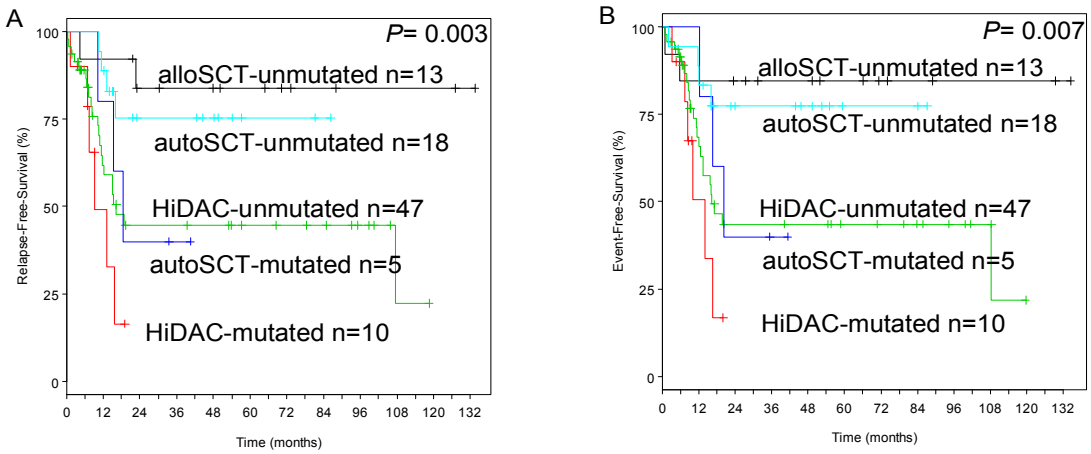

Figure 14. RFS (A) and EFS (B) according to the *FLT3*-TKD mutation status and different postremission therapies in 101 inv(16)-positive patients. alloSCT: allogeneic stem cell transplantation; autoSCT: autologous stem cell transplantation; EFS: event-free survival; HiDAC: high-dose cytarabine; RFS: relapse-free survival.

When looking for different treatment effects in the *KIT* exon 17 mutated patients, 6 subgroups were identified: alloSCT-*KIT*17-mutated (n = 2), alloSCT-*KIT*17-unmutated (n = 11), autoSCT-*KIT*17-mutated (n = 5), autoSCT-*KIT*17-unmutated (n = 18), HiDAC-*KIT*17-mutated (n = 4), HiDAC-*KIT*17-unmutated (n = 52). RFS and EFS was significantly different among the 6 groups (*P* < 0.0001 and *P* = 0.0007, respectively) (Figure 15). *KIT* exon 17 mutated patients undergoing alloSCT, or autoSCT had a better RFS and EFS than mutated patients treated with HiDAC. Similarly, unmutated patients treated by alloSCT or autoSCT had a better RFS and EFS compared to patients who received HiDAC. However, one should keep in mind, that the number of patients treated within the different regimens was quite small. There was no significant difference in OS among these groups (*P* = 0.26).

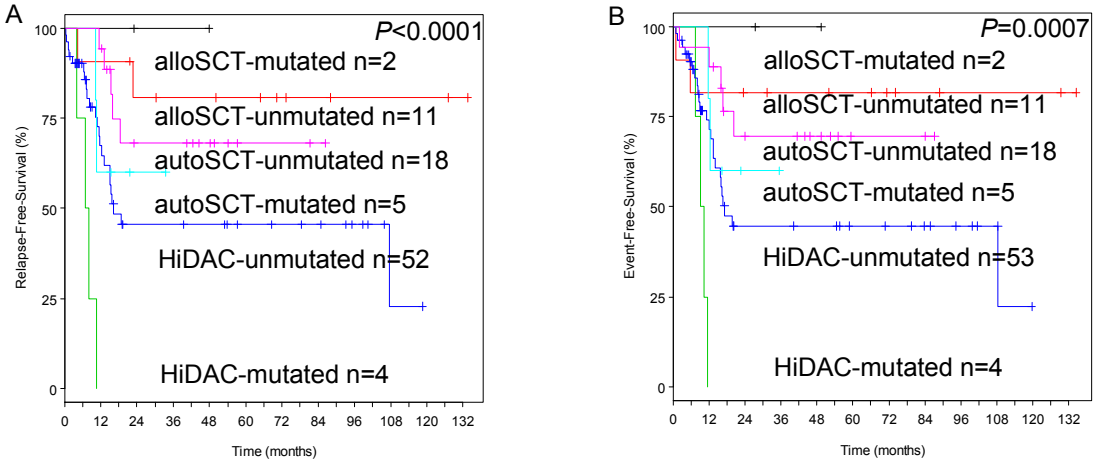

Figure 15. RFS (A) and EFS (B) of *KIT* exon 17 mutated patients according to different postremission therapies in 101 inv(16) patients. alloSCT: allogeneic stem cell transplantation; autoSCT: autologous stem cell transplantation; EFS: event-free survival; HiDAC: high-dose cytarabine; RFS: relapse-free survival.

Since *FLT3* and *KIT* mutations represent excellent targets for a therapy with tyrosine kinase inhibitors, we performed an explorative analysis for the combined *FLT3*-TKD and/or *KIT* exon 17 mutation status. Patients having either *FLT3*-TKD or *KIT* exon 17 or both mutations (P = 0.02) had a significantly inferior RFS compared to unmutated patients. However, there was no difference in OS which is mainly due to the high rate of second complete remission in this patient subgroup (P = 0.32) (Figure 16).

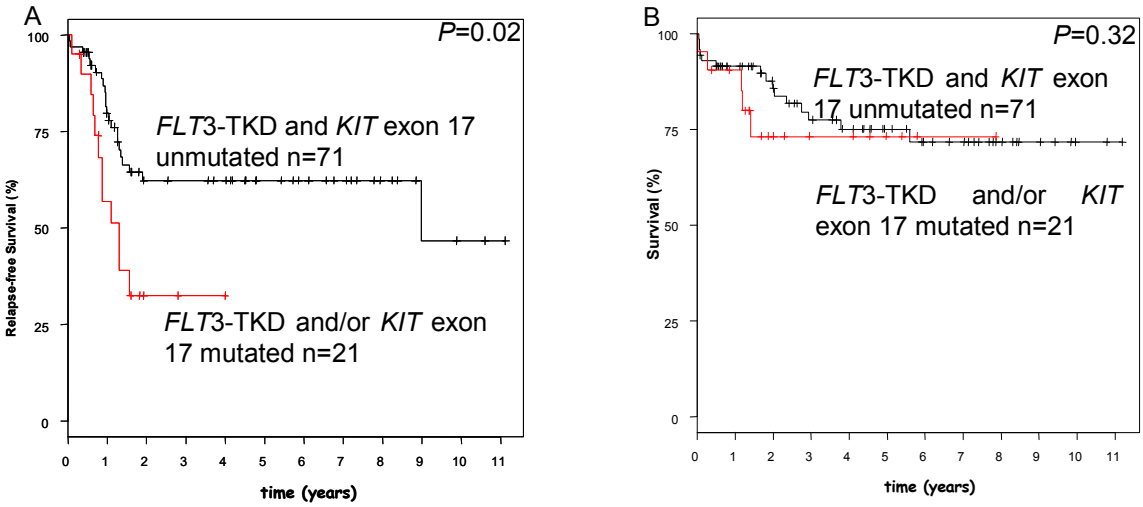

Figure 16. RFS (A) and OS (B) according to the combined *FLT3*-TKD and/or *KIT* exon 17 mutation status in 101 inv(16) patients. RFS: relapse-free survival; OS: overall survival.

3.5.1.1. Survival analyses for 94 younger inv(16)-positive patients

To address the different biological and clinical features of younger AML patients, the same survival analysis was performed in the 94 younger (< 60 years) inv(16)-positive patients. In analogy to the analysis of the whole cohort, univariate analysis showed a trend to reach a plateau after 1 year in RFS analysis (*P* = 0.64) for *NRAS* mutated patients. However, there was no significant difference in EFS, OS, and RFS in the younger inv(16)-patient group with regard to the *KRAS* and *KIT* mutation status.

Explorative subgroup analysis based on the *FLT3*-TKD and *KIT* exon 17 mutation status was performed for the different postremission therapies. None of the *FLT3*-TKD mutation patients underwent alloSCT. Comparing *FLT3*-TKD positive patients who received HiDAC or autoSCT with *FLT3*-TKD unmutated patients treated with the same approaches, *FLT3*-TKD mutated patients had an adverse RFS (*P* = 0.02) (Figure 17). *KIT* exon 17 mutated patients treated with HiDAC, had a significant inferior RFS (*P* = 0.00004) and OS (*P* = 0.05), in contrast to patients receiving alloSCT or autoSCT (RFS *P* = 0.70; OS *P* = 0.44) (Figure 18, 19). Therefore, the negative prognostic impact of *KIT* exon 17 mutations seems to be abrogated by SCT strategies, although the number of patients is quite small.

In analogy to the whole inv(16) group explorative subgroup analysis based on the combined *FLT3*-TKD and/or *KIT* exon 17 mutation status was performed. *FLT3*-TKD mutated and/or *KIT* exon 17 mutated patients had a significantly adverse RFS than unmutated patients (*P* = 0.01) whereas for the whole inv(6) cohort, there was no difference in OS (*P* = 0.33).

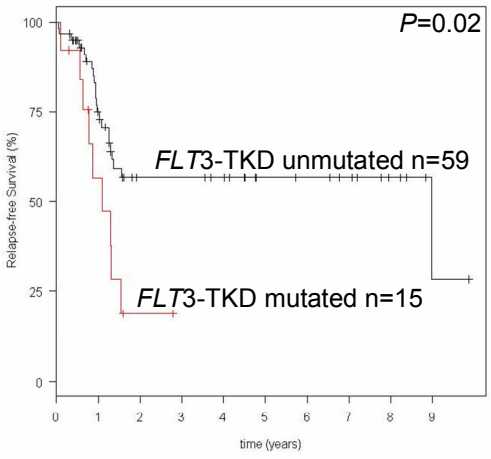

Figure 17. RFS according to the *FLT3*-TKD mutation status in 94 younger inv(16) patients treated with HiDAC or autoSCT regimen. autoSCT: autologous stem cell transplantation; HiDAC: high-dose cytarabine; RFS: relapse-free survival.

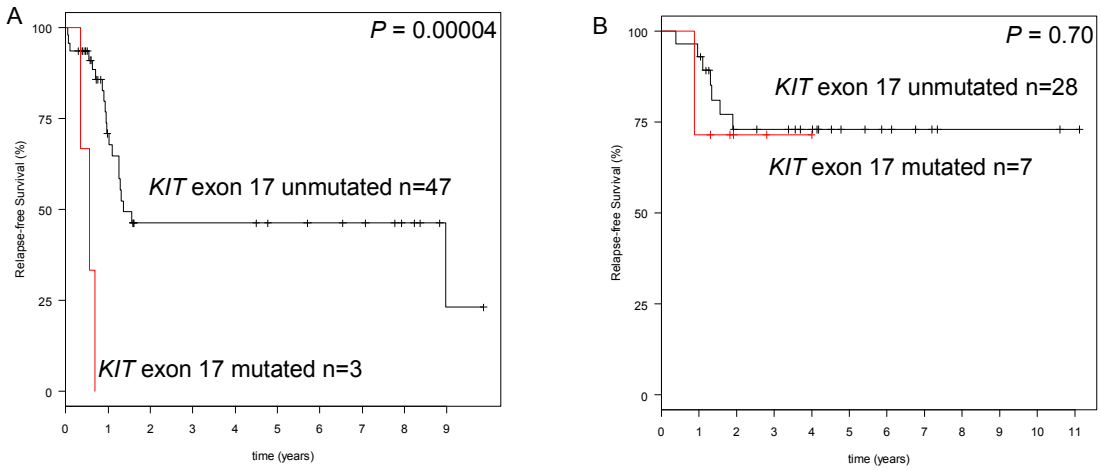

Figure 18. RFS according to the *KIT* exon 17 mutation status in 94 younger inv(16) patients treated with (A) HiDAC or (B) SCT regimens. SCT: stem cell transplantation; HiDAC: high-dose cytarabine; RFS: relapse-free survival.

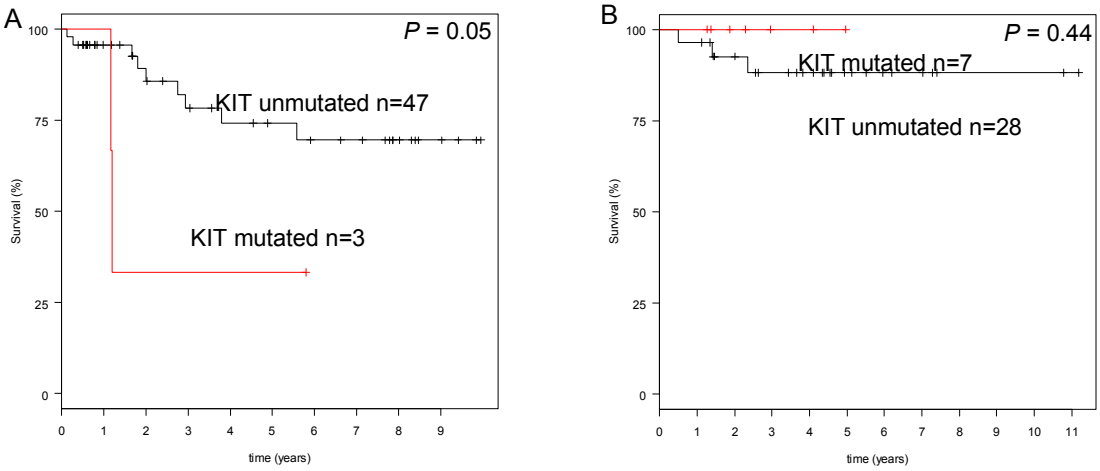

Figure 19. OS according to the *KIT* exon 17 mutation status in 94 younger inv(16)-positive patients treated with HiDAC (A) or SCT regimens (B). SCT: stem cell transplantation; HiDAC: high-dose cytarabine; OS: overall survival.

3.5.2. Survival analysis for t(8;21)-positive patients

Among 66 t(8;21)-positive patients, 52 younger patients received postremission therapy with HiDAC, and 14 older patients (≥ 60 years) were randomized for intensive postremission therapy with idarubicin and etoposide or

outpatient maintenance therapy. The median follow-up time for survival was 44 months (95%-CI, 36 - 57 months). In univariate analysis, *FLT3*-ITD mutations were significantly associated with an inferior RFS (*P* = 0.02), and in trend OS (*P* = 0.06), and EFS (*P* = 0.08) in all 66 patients (Figure 20). *KIT* mutated patients showed no significant difference in OS (*P* = 0.12) and RFS (*P* = 0.76) compared to the unmutated group. However, when looking at *KIT* exon 17 mutated patients in a subset analysis, these patients showed in trend a shorter OS (*P* = 0.06), whereas there was no difference in RFS (*P* = 0.49) and EFS (*P* = 0.18) for the whole t(8;21) group (Figure 21). With regard to the *NRAS* mutation status, there was no significant difference between mutated and unmutated patients in OS (*P* = 0.16), RFS (*P* = 0.43), and EFS (*P* = 0.32); the same was true for the *KRAS* mutations (OS *P* = 0.97, RFS *P* = 0.78, and EFS *P* = 0.32). However, the *NRAS* mutated group showed a trend to reach a plateau after 1 year of follow-up for OS, RFS and EFS (Figure 22). There was no significant difference in *FLT3*-TKD mutated and unmutated patients in OS (*P* = 0.82) and RFS (*P* = 0.38), and no difference in prognosis between *JAK2* mutated and unmutated patients. Since *FLT3*-ITD and *KIT* exon 17 mutations both represent potential targets for tyrosine kinase inhibitors, an explorative analysis combining both gene mutations versus unmutated patients was performed revealing a significant inferior OS (*P* = 0.006), EFS (*P* = 0.03), and in trend RFS (*P* = 0.06) (Figure 23).

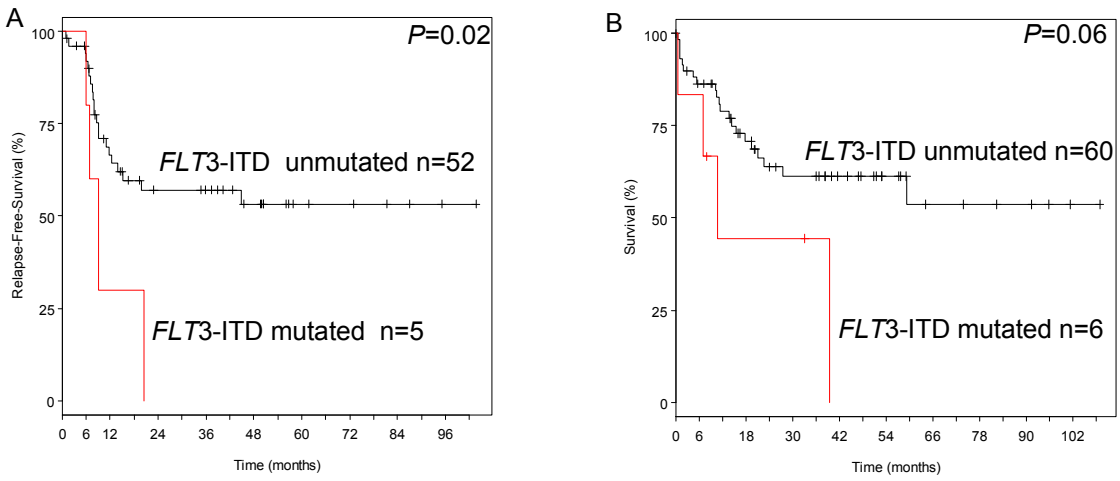

Figure 20. RFS (A) and OS (B) according to the *FLT3*-ITD mutation status in 66 t(8;21) patients. RFS: relapse-free survival; OS: overall survival.

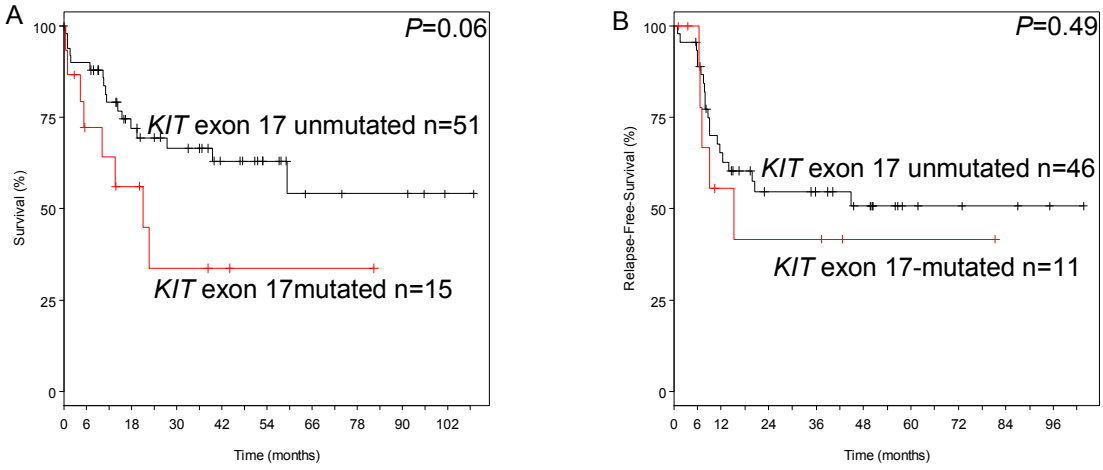

Figure 21. OS (A) and RFS (B) according to the *KIT* exon17 mutation status in 66 t(8;21) patients. RFS: relapse-free survival; OS: overall survival.

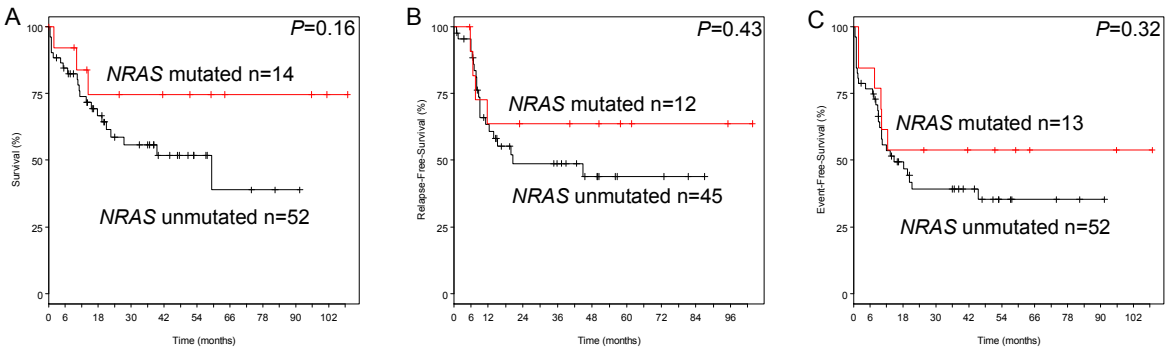

Figure 22. OS (A), RFS (B), and EFS (C) according to the *NRAS* mutation status in 66 t(8;21) patients. EFS: event-free survival; RFS: relapse-free survival; OS: overall survival.

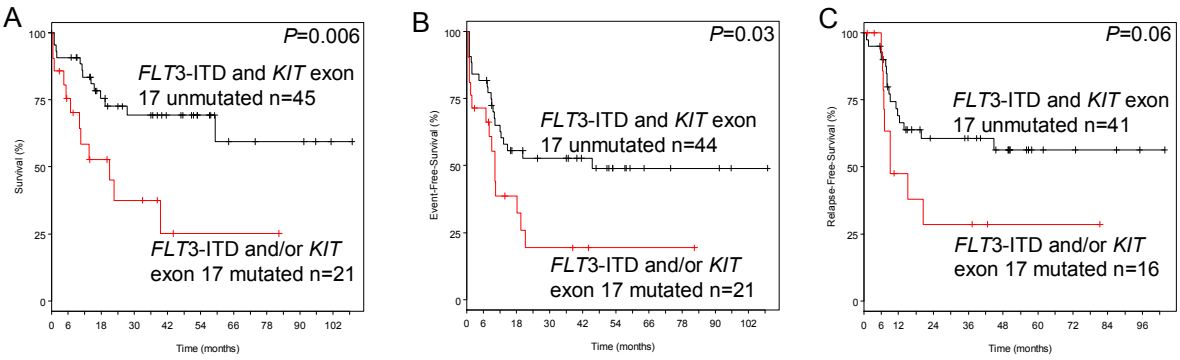

Figure 23. OS (A), EFS (B) and RFS (C) for the combined *FLT3*-ITD and/or *KIT* exon 17 mutation status in all t(8;21)-positive patients. EFS: event-free survival; RFS relapse-free survival; OS: overall survival.

3.5.2.1. Survival analyses for 52 younger t(8;21)-positive patients

In accordance with the inv(16)-positive group, we next analyzed the prognostic impact of *NRAS*, *KRAS*, *KIT, FLT3* and *JAK2* mutations in the 52 younger t(8;21) patients who were uniformly assigned to post-remission therapy with HiDAC. In univariate analysis, the adverse effect of *FLT3* mutations on prognosis (RFS P = 0.009, EFS P = 0.02, and OS P = 0.02) was mainly due to the presence of *FLT3*-ITD mutations. In contrast to the analysis of the whole t(8;21) patient cohort, in younger t(8;21)-positive patients *KIT* exon 17 mutations did not show significant differences in OS (P = 0.37), EFS (P = 0.31), and RFS (P = 0.27). In addition, there was no significant difference in OS, EFS and RFS for *KIT* mutated *NRAS* mutated *KRAS* mutated or *JAK2* mutated patients compared to the unmutated group. However, when combining *FLT3*-ITD and/or *KIT* exon 17 mutation status, patients with either one or both gene mutations showed a significantly adverse OS (P = 0.02), EFS (P = 0.02), and RFS (P = 0.01) compared to the unmutated patients.

4. Discussion

In this study, we evaluated the incidence and prognostic impact of *RAS*, *KIT*, *FLT3* and *JAK2* mutations in a large cohort of CBF leukemia consisting of 101 inv(16)-positive and 66 t(8;21)-positive AML patients. We demonstrated that these gene mutations were present in 86% of the 101 inv(16)-positive and in 58% of the 66 t(8;21)-positive patients, respectively. By using a comprehensive gene mutation screening approach, this is the first study revealing gene mutations with a frequency up to 90% in CBF AML (Care et al. 2003; Valk et al. 2004; Nanri et al. 2005; Beghini et al. 2004; Boissel et al. 2006; Cairoli et al. 2006). Moreover, correlation of these molecular findings with clinical outcome showed that some gene mutations are of prognostic relevance. In the inv(16)-positive group patients with *FLT3*-TKD and *KIT* mutations had a significantly worse outcome compared to unmutated patients. Likewise, in t(8;21)-positive group *FLT3*-ITD and *KIT* mutations were associated with a poor prognosis. The detection of gene mutations with such a high frequency can be attributed to the high sensitivity of DHPLC analysis that has been used in our study (Xiao et al. 2001).

Since there was no significant difference in mutation frequency and clinical outcome between the younger and the older inv(16)-positive or t(8;21)-positive patient groups for all gene mutation types (*RAS*, *KIT*, *FLT3*, and *JAK2*) that have been analyzed, the results of younger inv(16)- and t(8;21)-positive groups will not be discussed separately.

4.1. *RAS* analysis

Our analysis on a large cohort of inv(16)-positive AML identified *NRAS* mutations in 57%, which was considerably higher than the prevalence of 13% to 38% in CBF AML that have been described by other studies (Panagopoulos et al. 1996; Kiyoi et al. 1999; Bacher et al. 2006; Boissel et al. 2006). In accordance to other groups we also identified *NRAS* mutation to be the most detected gene mutation in inv(16) AML (Goemans et al, 2005; Boissel et al. 2006). Consistent with Bacher et al (2006) *NRAS* mutations mainly involved codon 61. In contrast, *KRAS* mutations were detected in 9% of our cohort which is less frequent than in the study from Bowen et al. (2005) who identified *KRAS* mutations in 23% of the inv(16)-positive patients. These findings may reflect a greater transforming capacity

for *NRAS* mutation in hematopoietic cells (Maher et al. 1994), and/or the predominance of *NRAS* P21 protein in myeloid cells (Furth et al. 1987) leading to a selective pressure for *NRAS* in comparison to *KRAS* gene mutation.

Among t(8;21)-positive patients, *NRAS* mutations were detected in 21% of the patients showing a higher incidence compared to previous reports (Boissel et al. 2006; Bowen et al. 2005). *KRAS* mutation were detected in 5% of all t(8;21)-positive patients, an incidence that is similar to the average frequency of *KRAS* mutations identified by other groups (Boissel et al. 2006; Goemans et al. 2006). Studies from Kiyoi et al. (1999) and Bacher et al. (2006) showed that *NRAS* mutations were significantly associated with lower peripheral leukocyte counts, whereas in our study no significant correlation of WBC could be identified for both the inv(16)- and t(8;21)-positive group, respectively. However, when considering the median percentage of BM and PB blast cells, there were significantly less BM blast cells in the *NRAS* mutated t(8;21)-positive cohort compared to the unmutated group.

Although *RAS* gene is frequently affected by mutation in CBF AML, the prognostic significance of this mutation in CBF AML is still under discussion. Boissel et al. (2006) did not observe *RAS* mutation affecting prognosis by screening 103 CBF AML (73 adults and 30 children CBF AML). In the study of Goemans et al. (2005), no significant influence on the clinical outcome was found in patients presenting *RAS* mutation including a small subset of 27 pediatric CBF AML. In the study from Bacher et al. (2006), on a large cohort of CBF leukemia, 113 inv(16)-positive and 116 t(8;21)-positive AML patients, no statistically significant impact of *NRAS* mutations was detected with regard to CR rate, OS and EFS. In addition, in the MRC AML 10 and AML 12 trial studies (Bowen et al. 2005), the presence of *NRAS* or *KRAS* mutations did not significantly influence prognosis when considering the specific risk groups inv(16)-positive and t(8;21)-positive AML. However, these historical data sets are hampered by the heterogeneity of patient treatment, and by limited patient numbers that do not allow to distinguish prognostic differences in CBF AML patients. In our study on a large series of inv(16)- and t(8;21)-positive patients, no significant differences in clinical outcome was observed between *NRAS* mutated and unmutated patients, which was in agreement with previous observations. However, in accordance to the study from

Bacher et al. (2006) Kaplan-Meier plot in our study showed a trend for *NRAS* mutated patients to reach a plateau after 1 year in both the inv(16) and the t(8;21)-positive group, respectively. For *KRAS* mutations there was no significant difference in clinical outcome between the mutated and the unmutated group in the inv(16)-positive as well as in the t(8;21)-positive patients, which might be possibly due to the low frequency of this mutation type.

4.2. *KIT* analysis

The incidence of *KIT* mutations in CBF leukemia has been reported to vary between 12.8% and 46.1% (Care et al. 2003; Kohl et al. 2005; Boissel et al. 2006; Schnittger et al. 2006). In our current study, *KIT* mutations were detected in 28% of the inv(16)-positive patient group; 18% of the 101 inv(16) patients exhibited a *KIT* exon 8 mutation, whereas 11% had exon 17 mutations. In the t(8;21)-positive group, *KIT* mutations were detected in 24%; exon 8 mutations (6%) were less common than in inv(16) patients, whereas exon 17 mutations (23%) were observed with a relatively high frequency compared to the inv(16) group. In accordance to previous findings (Gari et al. 1999; Care et al. 2003; Goemans et al. 2006; Boissel et al. 2006), we observed all different *KIT* exon 8 mutation types that have been described so far, like small deletions or deletions and insertions affecting the same codon D419. The majority of exon 17 mutations occurred at D816 and very rarely in N822, which is somewhat in contrast to the findings of Wang et al. (2005) who described the most frequent *KIT* exon 17 mutation in codon N822 in t(8;21) AML. These disparities may be possibly due to ethnic and geographic variations. Given the aforementioned genetic heterogeneity of *KIT* mutation types, it is crucial to determine the exact type of *KIT* mutation in each patient because activity of specific TK inhibitors have been reported to be dependant on the *KIT* mutation type (Paschka et al. 2006).

In agreement with the report from Paschka et al. (2006), in our study inv(16)-positive patients with *KIT* mutations had significantly higher PB blast cells compared to the *KIT* unmutated cases. The same was true when comparing exon 17 mutated patients with those having *KIT* wild-type. In addition, in our study, *KIT* mutated t(8;21)-positive patients had significantly higher LDH level than unmutated patients, and the same results showed up when performing subgroup analysis for

KIT exon 17 mutations.

To date, several studies have analyzed the prognostic significance of *KIT* mutations in inv(16)-positive AML. However, the prognostic significance of this gene mutation is discussed controversially. Care et al. (2003) showed that *KIT* exon 8 mutations are associated with an increased relapse rate but have no impact on OS in 63 inv(16)-positive AML; while data from a CALGB (Paschka et al. 2006) on 61 adult inv(16)-positive AML patients demonstrated that the presence of *KIT* exon 17 mutations conferred a higher relapse risk, and both exons 8 and 17 mutations appeared to adversely affect OS. In contrast to these findings both Cairoli et al. (2006) and Boissel et al. (2006) were unable to show a prognostic impact of *KIT* mutations in 25 adult inv(16)-positive patients and in 47 adult and childhood inv(16)-positive cases, respectively. However, these studies were heterogeneous with respect to number of patient that have been analyzed, treatment, various techniques that have been used for mutation screening, and parts of the *KIT* gene, which have been screened. In a recent report from Schnittger et al. (2006), *KIT* gene D816 mutations have been analyzed in a relative large number of inv(16) patients (n = 97) by using a melting curve-based Light Cycler assay. In their study, only two patients harboring the *KIT* D816 mutation have been identified not allowing to perform a meaningful clinical correlation.

In our study, to our knowledge the largest inv(16) AML cohort that has been analyzed so far, univariate analysis for RFS and OS did not reveal a significant difference between patients with and without *KIT* mutations and this was also the case when comparing *KIT* exon 17 mutated and *KIT* exon 17 wild-type patients. However, when correlating the *KIT* exon17 mutation status according to the different types of postremission therapy, our data suggest that patients with *KIT* exon 17 mutations might benefit from more intensive postremission strategies such as allogeneic or autologous SCT as indicated by an improved RFS and reduced relapse risk compared to patients who received consolidation therapy with HiDAC. Thus, the adverse prognosis of *KIT* exon 17 might be overcome by the more intensive SCT strategies. However, the current study was performed retrospectively with only 4 *KIT* exon 17 mutated patients have been treated with HiDAC chemotherapy. Therefore, the role of pretreatment *KIT* exon 17 mutation status in the context of different postremission regimens is intriguing but has to be

evaluated in larger prospective trials.

Recently, several studies evaluated the prognostic impact of *KIT* mutations in t(8;21)-positive AML. Schnittger et al. (2006), who analyzed 76 adult t(8;21) AML patients with *KIT* exon 17 for D816 mutations, found an independent negative impact on EFS and OS. Data from an Italian retrospective study on 42 t(8;21)-positive patients (Cairoli et al. 2006) showed that patients with *KIT* D816 mutations had a significantly higher incidence of relapse and a lower OS. In accordance to these findings the France research group (Boissel et al. 2006) reported on 50 adult and childhood t(8;21)-positive leukemia and showed that patients with *KIT* exon 17 mutations have an adverse OS and EFS. Paschka et al. (2006) demonstrated that *KIT* mutations are associated with a higher CIR, but do not impact on survival. In analogy to the studies on inv(16)-positive leukemia, studies on the prognostic impact of *KIT* mutations in t(8;21)-positive AML are limited due to the size of patient population, the heterogeneity of the patients and the treatment and the parts the *KIT* gene was screened for; in particular, some studied just focused on *KIT* D816 mutations.

In contrast to previous findings, in our study univariate analysis showed no significant difference between *KIT* mutated and unmutated patients for RFS, EFS and OS. The same was true when focusing on *KIT* exon 17 mutated cases. This might be related to differences in patient numbers, distinct treatment regimens, in particular the type of salvage therapy, as well as differences in ethnic background. Finally, one have to take into account that other molecular aberrations that have not been identified yet might contribute to the differences in the clinical phenotype.

4.3. *FLT3* analysis

The prevalence of *FLT3*-ITD mutations in CBF leukemia varies from one study to another. Care et al. (2003) reported 3% *FLT3*-ITD mutations in 63 inv(16)-positive patients, and 4% in 47 t(8;21)-positive patients, respectively. In the study of Boissel et al. (2006), only one t(8;21)-positive patient had a *FLT3*-ITD mutation out of 103 adult and childhood CBF leukemia patients. Schessl et al. (2006) detected 11 (8%) *FLT3*-ITD mutated patients in a cohort of 135 t(8;21)-positive patients. In comparison to previous reports, in our study, we have screened a large cohort of CBF patients and found the incidence of *FLT3*-ITD was

to be 4% in the inv(16)-positive group, and 9% in the t(8;21)-positive group.

In contrast *FLT3*-ITD, *FLT3*-TKD mutations has been detected with a higher frequency in inv(16)-positive AML; whereas the incidence is low in t(8;21)-positive leukemia. Care et al. (2003) identified *FLT3*-TKD mutation 5% of 63 inv(16)-positive and in and 2% of 47 t(8;21)-positive patients, respectively. Boissel et al. (2006) identified *FLT3*-TKD mutation in 6% of 47 inv(16)-positive patients and in 7% of 56 t(8;21)-positive patients, respectively. However, this study also included 30 cases of childhood leukemia. Schessl et al. (2006) detected *FLT3*-TKD mutations in 2% of 135 t(8;21)-positive patients. In a recent study published by Mead and colleagues (2007) a high frequency of 24% *FLT3*-TKD mutations (13/55) was reported in cases with inv(16); whereas the incidence in the t(8;21)-positive group was 7% (5/69). In our study, *FLT3*-TKD mutations were detected in 16% of the inv(16)-positive leukemia and in 5% of the t(8;21)-positive group, respectively.

Obviously, the frequency of *FLT3*-ITD and *FLT3*-TKD mutations in CBF AML is markedly different in comparison to normal karyotype AML where *FLT3*-ITD mutations are relatively common (25 - 30%) and *FLT3*-TKD occur with an incidence of 8% to 12% (Small D. 2006). In comparison to most others studies in CBF AML, in our study, we identified a relatively high incidence of 19% of *FLT3* (ITD and TKD) mutations in inv(16)-positive patients and 14% in t(8;21)-positive patients. It is very unlikely that these differences are caused by technical reasons but are rather due to the relatively large size of patients number tested.

In normal karyotype AML *FLT3*-ITD mutations have clearly emerged as a negative prognostic marker (Yamamoto et al. 2001; Thiede et al. 2002; Fröhling et al. 2002), whereas more recent data provide evidence that *FLT3*-TKD mutations are associated with an improved clinical outcome (Mead et al. 2007). However, the prognostic impact of either *FLT3*-ITD or *FLT3*-TKD mutations in CBF leukemia is still under discussion. In the study by Care et al. (2003) *FLT3*-ITD or *FLT3*-TKD mutations did not impact the prognosis of inv(16)-positive and t(8;21)-positive leukemia. Boissel et al. (2006) found *FLT3*-ITD and *FLT3*-TKD to be associated with a significantly worse EFS and OS in inv(16)- and t(8;21)-positive patients. However, in their study of 103 CBF AML, a significant number of childhood leukemia was analyzed.

In contrast to previous results, in our study, *FLT3*-TKD mutations were significantly associated with an inferior RFS and in trend with an adverse EFS in the inv(16)-positive group, whereas for *FLT3*-ITD mutations we could not identify a prognostic impact. However, the number of *FLT3*-ITD mutations was quite low with only 4 mutated patients in the whole inv(16) group. For the t(8;21)-positive group the presence of *FLT3*-ITD mutations was associated with a significantly inferior RFS and in trend with a shorter OS and EFS. In contrast, *FLT3*-TKD mutations did not show a prognostic significance.

Since *FLT3* gene (ITD and TKD) mutations and *KIT* exon 17 mutations both represent potential targets for tyrosine kinase inhibitors, an explorative combined analysis for either *FLT3*-TKD and/or *KIT* exon 17 mutated patients versus unmutated patients was performed revealing a significant inferior RFS in inv(16) patients, and a significant inferior OS, EFS, and in trend RFS in t(8;21)-positive patients. There was no effect on OS in inv(16)-positive cases, which is mainly due to the fact that almost all inv(16)-positive patients achieved a second CR.

4.4. *JAK2* analysis

Recently, several studies have described *JAK2* mutations as cooperating mutations in t(8;21)-positive AML (Lee et al. 2006; Döhner et al. 2006; Schnittger et al. 2007). In the study of Lee et al. (2006), *JAK2* mutations were found in two of 12 t(8;21)-positive patients. Schnittger et al. (2007) identified *JAK2* mutations in two (8%) of the 24 t(8;21)-positive cases. *JAK2* V617F mutations were also detected by Illmer and colleagues (2007) who screened 138 CBF leukemia patients with an incidence of 3.5% (n = 5); in the t(8;21)-positive group 2 patients harbored the mutation and in the inv(16) group 3 cases were *JAK2* V617F positive. In our series, *JAK2* mutations were exclusively detected in t(8;21)-positive AML with an incidence of 6% (4/66). In contrast to the report from Illmer et al. (2007) who demonstrated *JAK2* V617F mutations were associated with an aggressive clinical outcome in CBF leukemia, the four mutated t(8;21) patients in our study had no distinct biological or clinical features compared to the unmutated t(8;21) patients. However, in both studies the number of *JAK2* mutated patients was quite small not allowing any conclusions on the prognostic impact of *JAK2* mutations in this AML subgroup. Future studies on larger cohorts might facilitate the evaluation of the

prognostic impact of *JAK2* V617F mutations in CBF leukemias.

4.5. Conclusion

In the current study, mutation screening of the genes encoding for *RAS*, *KIT*, *FLT3*, and *JAK2* revealed a mutation rate of almost 90% in inv(16)-positive patients and almost 60% in the t(8;21)-positive cohort, respectively. This data suggest that activating mutations in the *RTK* and in the *RAS* pathways might play an important role in the pathogenesis of CBF AML.

Recent data from murine models showed that the expression of one single gene mutation is not sufficient to cause AML and that cooperation of several genes is necessary. Based on this observation a model of two complementation groups of mutations has been suggested. The class I mutations leading to activation of signaling pathways resulting in enhanced proliferation and/or survival of leukemic cells and the class II mutations affecting transcription factors or components of the transcriptional-coactivation complex leading to an impaired differentiation of hematopoeitc cells. The fusion transcripts *CBFβ-MYH11* or *AML1-ETO* resulting from inv(16) or t(8;21), respectively, have been shown to belong to the class II mutation group, whereas mutations in the *RTK* genes such as *FLT3, KIT, or JAK2* or mutations in the *RAS* gene fall into the group of class I mutations. Therefore, it is very likely that cooperation of these gene mutations might be one of the major pathogenic events in CBF leukemia. Our data provide further evidence that some of these secondary gene mutations are of prognostic relevance. In our study *FLT3*-TKD and *KIT* mutations were associated with an inferior outcome in inv(16)-positive AML. Likely, *FLT3*-ITD and *KIT* mutations were associated with an unfavorable prognosis in t(8;21)-positive patients. In addition, in the inv(16) group our data suggest that different consolidation regimens may impact on the prognosis dependent on the *FLT3*-TKD and *KIT* mutation status. However, the number of mutated patients who received HiDAC or an intensive SCT-based regimen in our study was quite small. This study provides an important basis to fully evaluate the role of SCT in *KIT* and/or *FLT3* mutated patients in a larger number of homogenously treated CBF patients.

Identification of secondary gene mutations in CBF leukemia is of major clinical relevance since some of these molecular rearrangements can be targeted by

molecular therapies as it has been impressively shown for CML. Activated *RTK* pathways can be inhibited by a number of tyrosine kinase inhibitors (Weisberg et al. 2002) that have recently been developed. Likewise, the *RAS* pathway can be successfully targeted by Farnesyltransferase-inhibitors (Lancet et al. 2003).

Based on our data that were generated within one of the largest cohorts of CBF leukemias, together with data reported by several other groups, *FLT3* and *KIT* gene mutations seem to be of prognostic significance. In the near future, mutation screening of CBF leukemias might reach clinical importance, in particular for the identification of patients who might benefit from molecular targeted therapies.

5. Summary

Patients with core binding factor (CBF) acute myeloid leukemia (AML), defined by the inversion/translocation inv(16) and the translocation t(8;21) [inv(16)(p13q22)/t(16;16)(p13;q22) and t(8;21)(q22;q22)] are considered to have a favorable outcome, in particular when treated with cytarabine-based consolidation regimens. However, a significant proportion of these patients relapse and survival after 5 years is approximately 50%. These findings together with studies from murine models suggest that additional genetic lesions are underlying the clinical heterogeneity of CBF leukemias. The recently described mutations in the signaling molecules *RAS*, *KIT*, *FLT3*, and *JAK2* represent potential secondary genetic lesions that might contribute to leukemic transformation through their constitutive activation.

In this study we determined the incidence of *RAS* (*NRAS* and *KRAS* exon 1 and exon 2), *KIT* (exons 8, 10, 11, and 17), *FLT3* [Internal tandem duplication (ITD) and Tyrosine kinase domain (TKD) at codon D835], and *JAK2* V617 mutations in 101 adults patients with inv(16) (median age 41 years) and 66 adult patients (median age 49 years) with t(8;21)-positive AML, respectively, and evaluated their prognostic impact on clinical outcome.

KIT and *RAS* mutation screening was performed using a sensitive Denaturing high performance liquid chromatography (DHPLC)-based assay; *FLT3*-ITDs were identified by Polymerase chain reaction (PCR), and detection of *FLT3*-TKD mutation was performed by PCR and restriction fragment length polymorphism (PCR-RFLP). The presence of the *JAK2* V617 mutation was revealed by allele-specific PCR. All samples with abnormal DHPLC profile and specific bands in gel were confirmed by direct sequencing. Patients were enrolled in 5 AML Study Group (AMLSG) treatment trials [AML HD93, AML HD98-A, AML HD98-B, AMLSG 07-04, and AMLSG 06-04] and the Hemato-Oncologie voor Volwassenen Nederland (HOVON) protocol. Postremission therapy in t(8;21)-positive AML was consistently high-dose cytarabine (HiDAC) in all trials, whereas in inv(16) AML cohort of patients were treated with HiDAC, autologous or allogeneic stem cell transplantation (SCT) in first complete remission (CR). The comprehensive mutation analyses of the *RAS*, *KIT*, *FLT3, or JAK2* genes revealed a high mutation rate of 86% with highest frequencies in *NRAS* (51%), followed by *KIT* (28%) and

FLT3-TKD (16%) mutations; in particular, 10/28 *KIT* mutations affected exon 17. *KRAS* and *FLT3*-ITD mutations were detected in 9% and 4%, respectively. In analogy, for the t(8;21)-positive patients 58% exhibited gene mutations in at least one of the genes. Highest frequencies were detected for *KIT* mutations (24%) followed by *NRAS* (21%) and *FLT3*-ITD mutations (9%); 10/16 *KIT* mutations affected exon 17. *KRAS*, *FLT3*-TKD and *JAK2* mutation were detected in 5%, 5%, and 6%, respectively. In the inv(16) cohort, univariate analyses showed a significant inferior relapse-free survival RFS (P = 0.004) for patients with *FLT3*-TKD, whereas for the other gene mutations there was no significant difference between mutated and unmutated patients. Interestingly, when performing survival analysis for the type of postremission therapy according to the *FLT3*-TKD and *KIT* exon 17 mutation status, we saw a benefit for mutated patients who received a more intensive treatment regimen like autologous or allogeneic SCT in comparison to patients undergoing HiDAC. However, one has to keep in mind that the patient number in the different treatment arms is still quite small. Likely, *FLT3*-ITD and *KIT* mutations were associated with an unfavorable prognosis in a large series of t(8;21)-positive patients. The negative prognostic impact of *FLT3* and *KIT* mutations in CBF leukemia was clearly demonstrated by an explorative analysis using the combined mutation status [*FLT3*-TKD and/or *KIT* exon 17 for the inv(16)-positive group; *FLT3*-ITD and/or *KIT* exon 17 for the t(8;21)-positive group] versus the unmutated patients. In the inv(16)-positive group, patients with either one of the two mutation types had a significantly inferior RFS (P = 0.02) than the wild-type patients. In the t(8;21)-positive group, RFS and overall survival (OS) were significantly worse for patients exhibiting a *FLT3*-ITD and/or *KIT* exon 17 mutation (P = 0.06 and P = 0.006).

In summary, the high frequency of *RAS*, *KIT*, *FLT3* or *JAK*2 gene mutations in CBF leukemia further sustain the model of different cooperating groups of gene mutations. In our study *FLT3* and *KIT* exon 17 mutations seems to be of prognostic relevance. Although we screened one of the largest series of CBF leukemias, the patient number for subgroup analysis is still small. Therefore, our preliminary findings on the clinical benefit for inv(16)-positive patients receiving SCT-based treatment have to be evaluated on a larger patient population. In addition, information on the *KIT* and *FLT*3 mutation status might soon reach clinical importance for identifying patients who are eligible for molecular targeted therapies.

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Du J, Schlenk RF, Corbacioglu A, Habdank M, Scholl C, Fröhling S, Bullinger L, Ganser A, Döhner H, and Döhner K: *RAS*, *KIT* and *FLT3* Gene Mutations in inv(16)/t(16;16)-Positive Acute Myeloid Leukemia (AML): Incidence and Relevance on Clinical Outcome. 48th Annual Meeting of the American Society of Hematology (ASH), December 09-12, 2006, Orlando, FL, USA (Abstract in Blood 108(11):652a 2006)

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