Molecular and flow cytometric diagnostics for evaluation of therapy efficacy in myeloid leukemias

Nancy Boeckx

MOLECULAR AND FLOW CYTOMETRIC DIAGNOSTICS FOR EVALUATION OF THERAPY EFFICACY IN MYELOID LEUKEMIAS

Moleculaire diagnostiek en flowcytometrische immunofenotypering ter evaluatie van therapie-effectiviteit bij patiënten met myeloïde leukemie

ISBN 90-73436-73-7

MOLECULAR AND FLOW CYTOMETRIC DIAGNOSTICS FOR EVALUATION OF THERAPY EFFICACY IN MYELOID LEUKEMIAS

Moleculaire diagnostiek en flowcytometrische immunofenotypering ter evaluatie van therapie-effectiviteit bij patiënten met myeloïde leukemie

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden
op woensdag 22 maart 2006 om 15.45 uur

door

Nancy Boeckx

geboren te Mortsel, België

PROMOTIECOMMISSIE

Promotoren:
Prof.dr. J.J.M. van Dongen
Prof.dr. P. Vandenberghe

Overige leden:
Prof.dr. R. Pieters
Prof.dr. B. Löwenberg
Prof.dr. G. Verhoef

Copromotor:
Dr. Ph. Vandekerckhove

The studies described in this thesis were performed at the Department of Immunology, Erasmus MC, Rotterdam, The Netherlands and at the Department of Laboratory Medicine, Hematology, University Hospital Gasthuisberg, Leuven, Belgium.

Illustrations : Marieke Comans-Bitter and Tar van Os
Printing : Haveka B.V., Alblasserdam
Cover : Tar van Os and Maria Everix
Lay-out : Wendy Netten, Marcia IJdo-Reintjes, Daniëlle Korpershoek and Erna Moerland-van Eenennaam

MOLECULAR AND FLOW CYTOMETRIC DIAGNOSTICS FOR EVALUATION OF THERAPY EFFICACY IN MYELOID LEUKEMIAS

Moleculaire diagnostiek en flowcytometrische immunofenotypering ter evaluatie van therapie-effectiviteit bij patiënten met myeloïde leukemie

CONTENTS

Part 1.	**General introduction**	9
Part 2.	**Fusion genes and immunoglobulin and T-cell receptor gene rearrangements as minimal residual disease targets in myeloid leukemias**	67
2.1	Fusion genes and junctional regions of rearranged immunoglobulin/T-cell receptor genes as PCR-targets for detection of minimal residual disease	69
2.2	Fusion gene transcripts and Ig/TCR gene rearrangements are complementary but infrequent targets for PCR-based detection of minimal residual disease in acute myeloid leukemia *Leukemia 2002; 16: 368-375*	75
2.3	Differential stability of control gene and fusion gene transcripts over time may hamper accurate quantification of minimal residual disease – a study within the Europe Against Cancer Program *Leukemia 2004; 18: 884-886*	93
2.4	Identification of e19a2 *BCR-ABL* fusions (μ-BCR breakpoints) at the DNA level by ligation-mediated PCR *Leukemia 2005; 19: 1292-1295*	101

Part 3. **Clinical applications of molecular and flow cytometric diagnostics in myeloid leukemias** 109

3.1 Clinical applications of molecular and flow cytometric diagnostics in detecting minimal residual disease and evaluating therapy efficacy in myeloid leukemia 111

3.2 An inv(16)(p13q22) positive acute myeloid leukemia relapsing as acute precursor B-cell lymphoblastic leukemia
Haematologica 2004; 89: ECR28 117

3.3 Quantification of *CBFB-MYH11* fusion gene levels in paired peripheral blood and bone marrow samples by real-time PCR
Leukemia 2005; 19: 1988-1990 127

3.4 Imatinib mesylate induces durable complete remission of advanced CML persisting after allogeneic bone marrow transplantation
Leukemia 2003; 17: 458-460 135

3.5 High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg®) treatment in acute myeloid leukemia patients
Leukemia 2004; 18: 983-988 143

Part 4. **Concluding remarks and future perspectives** 155

Abbreviations 169

Summary 171

Samenvatting 175

Dankwoord 181

Curriculum vitae 183

Publications 185

PART 1

General introduction

Acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) are clonal expansions of myeloid cells in the bone marrow, blood or other tissues. The incidence of AML and CML is shown in Figure 1. AML occurs at any age group, but its incidence increases with age. The vast majority of AML occurs in adults, especially at >60 years of age. CML also occurs in all age groups and shows an increasing incidence with age. Only 5 to 10% of CML cases occur in patients younger then 20 years of age. The median age of CML at diagnosis is in the fifth and sixth decades of life.

In the first section, the immunophenotypic characteristics, molecular genetic abnormalities and immunogenotypic characteristics of AML and CML are discussed. Standard therapy and some innovative treatment modalities of myeloid leukemias are reviewed in the second section. Finally, quantitative techniques for the detection of minimal residual disease (MRD) and the clinical significance of MRD monitoring in AML and CML are discussed.

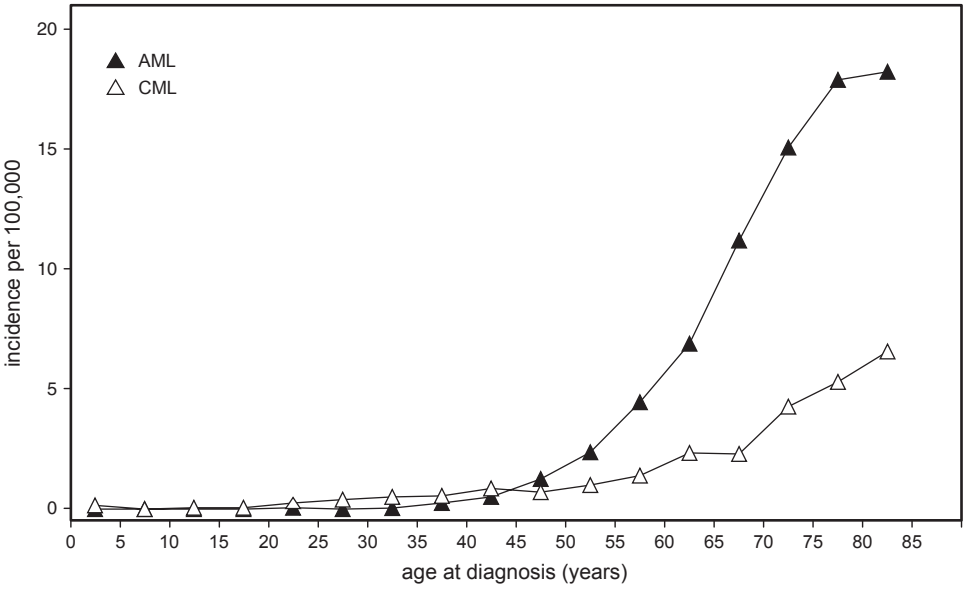


Figure 1. Age-specific incidence of AML (closed symbols) and CML (open symbols) in the Netherlands (1994-1998).

1. IMMUNOPHENOTYPE AND MOLECULAR CHARACTERISTICS OF MYELOID LEUKEMIAS

1.1 IMMUNOPHENOTYPE OF MYELOID LEUKEMIA

Immunophenotypic characteristics of myeloid leukemia

The tumor cells in acute leukemia are generally regarded as the malignant counterparts of cells in immature and more mature stages of hematopoiesis. Analysis of several cellular parameters by flow cytometry enables the determination of the differentiation stage of normal and abnormal myeloid cell populations. During myeloid differentiation, more or less characteristic markers appear on the cell surface membrane, while others disappear.[1-4] A hypothetical scheme of myeloid differentiation is shown in Figure 2.

By combining the information of several parameters including cell size, granularity, and expression levels of surface and intracellular molecules, it is possible to identify an immunophenotypic signature of the leukemic cells.[5,6] The immunophenotypes of the AML subtypes according to the French-American-British (FAB) classification and the corresponding classes of the World Health Organization (WHO) are summerized in Table 1.[7-11] Virtually all AML subtypes are positive for CD13 and/or CD33 and the majority of AML also expresses CD65 and cytoplasmatic myeloperoxidase.[12-14] Markers used for additional characterization are CD11b, CD11c, HLA-DR, CD14, CD15 (particularly myeloid/monocytic lineage), CD36, CD71, and CD235a (erythroid lineage), and CD41, CD42 and CD61 (megakaryocytic lineage).[3,15,16] HLA-DR expression is negatively associated with AML-M3.[13,17] The majority of $CD34^+$ AML blasts co-express the CD133 antigen.[18,19] An example of flow cytometric immunophenotyping of an AML at diagnosis is given in Figure 3.

Leukemia associated immunophenotypes in myeloid leukemia

The discrimination between leukemic myeloid cells and normal bone marrow myeloid cells is based on so-called leukemia-associated immunophenotypes (LAIP).[20,21] These immunophenotypes are either aberrant or extremely uncommon within normal bone marrow and therefore allow the discrimination between the leukemic and normal cells. These LAIP's in AML generally involve: a) cross-lineage antigen expression (i.e. the expression of typical lymphoid markers on myeloid cells), b) asynchronous expression of antigens (i.e. the co-expression of early markers such as CD34 or CD117 with antigens associated with advanced maturational stages of myeloid differentiation including CD15, CD56 or CD11b/c), c) antigen over- or underexpression (i.e. the presence of antigens such as CD13 or CD33 on leukemic cells at abnormally high or low levels), and d) ectopic antigen expression. Ectopic antigen expression in AML refers to the expression of antigens normally only

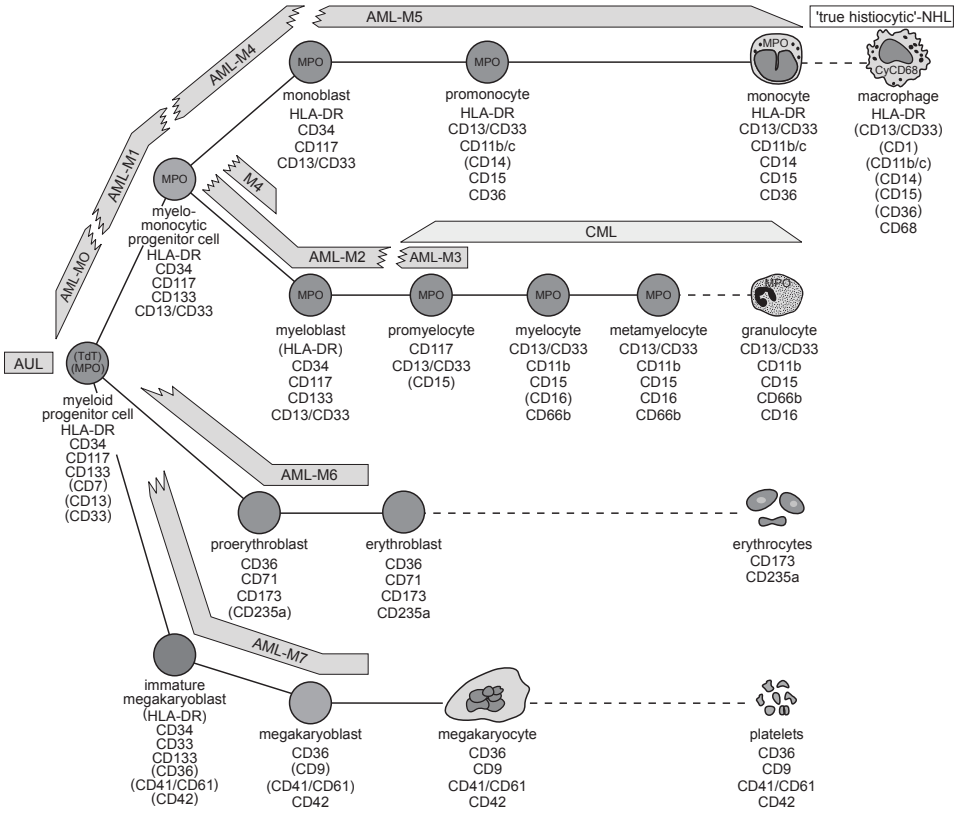


Figure 2. Hypothetical scheme of myeloid differentiation.
The expression of relevant immunologic markers is indicated for each differentiation stage; markers in parentheses are not always expressed. The bars represent the various types of leukemias. Abbreviations used: AUL = acute undifferentiated leukemia, AML = acute myeloid leukemia (M0 to M7; classification according to the "French-American-British" criteria); CML = chronic myeloid leukemia; NHL = non-Hodgkin's lymphoma.

expressed on non-hematopoietic cells, such as expression of the NG2 antigen in a subset of AML with *MLL* gene rearrangements.[22-24] Cross-lineage expression in AML usually concerns the expression of CD2, CD7, CD19, or terminal deoxynucleotidyl transferase (TdT) and can be found in up to one third of adult AML[13,20,25-27] and up to two-third of childhood AML (Van der Velden, personal communication).

Using an appropriate panel of monoclonal antibodies, LAIP's can be defined in about 80% of AML, with the majority of cases having at least two aberrant

Table 1. Immunophenotypic characteristics of AML.

AML subtype / Marker	AML-M0[a]	AML minimally differentiated[b]	AML-M1[a]	AML without maturation[b]	AML-M2[a]	AML with maturation[b]	AML-M3[a]	AML with t(15;17) (q22;q12) (*PML-RARA*)[b]
CD34	++		++		+		±	
CD117	++		++		++		+	
CD133	++		+		+		-	
HLA-DR	++		++		+		-	
TdT	+		+		+		±	
CD13/33	++		++		++		++	
CD65	±		+		+		+	
MPO	+ / ++		+		++		++	
CD11b/c	- / ±		- / ±		- / ±		-	
CD14	-		-		-		-	
CD15	±		±		++		±	
CD36	-		-		-		-	
CD235a	-		-		-		-	
CD41/CD61	-		-		-		-	
CD42	-		-		-		-	

[a] AML subgroups according to the French-American-Britisch (FAB) classification[7]
[b] AML subgroups according to the World Health Organization (WHO) classification[10]
cut-off for positivity ≥ 20% (cut-off for positivity of MPO ≥10%)[9]

-, <10% of the leukemias are positive; ±, 10-25% of the leukemias are positive; +, 25-75% of the leukemias are positive; ++, >75% of the leukemias are positive. The FAB and WHO classifications incorporate the immunophenotypic results for the recognition of AML-M0 (acute myeloid leukemia, minimally differentiated) and AML-M7 (acute megakaryoblastic leukemia). For the other AML subtypes, there is a moderate to fair correlation with specific markers.

Table 1. Immunophenotypic characteristics of AML (continued).

AML subtype / Marker	AML-M4[a]	Acute myelo-monocytic leukemia[b]	AML-M5a/b[a]	Acute monoblastic/ monocytic leukemia[b]	AML-M6[a]	Acute erythroid leukemia[b]	AML-M7[a]	Acute megakaryo-blastic leukemia[b]
CD34	±		+ / ±		+		+	
CD117	+		+		+		+	
CD133	±		-		-		-	
HLA-DR	++		++		+		++	
TdT	+		+		+		±	
CD13/33	++		++		+		++	
CD65	++		++		±		±	
MPO	++		++		+		-	
CD11b/c	++		++		-		-	
CD14	+		+ / ++		-		-	
CD15	-		-		-		-	
CD36	+		+		++		+	
CD235a	-		-		+		-	
CD41/CD61	-		-		-		++	
CD42	-		-		-		+	

[a] AML subgroups according to the French-American-Britisch (FAB) classification[7]
[b] AML subgroups according to the World Health Organization (WHO) classification[10]
cut-off for positivity ≥ 20% (cut-off for positivity of MPO ≥10%)[9]

-, <10% of the leukemias are positive; ±, 10-25% of the leukemias are positive; +, 25-75% of the leukemias are positive; ++, >75% of the leukemias are positive. The FAB and WHO classifications incorporate the immunophenotypic results for the recognition of AML-M0 (acute myeloid leukemia, minimally differentiated) and AML-M7 (acute megakaryoblastic leukemia). For the other AML subtypes, there is a moderate to fair correlation with specific markers.

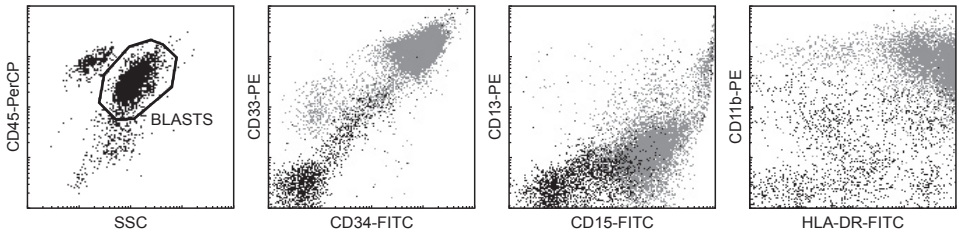


Figure 3. Flow cytometric immunophenotyping of whole bone marrow from a patient with an AML-M5 at diagnosis.
The CD34/CD33, CD15/CD13, and HLA-DR/CD11b stainings were analyzed for the indicated CD45/SSC gated blast cell population. The myeloid blast cells expressed CD33, CD34 (strong expression), CD13 (weak expression), CD15, CD11b, HLA-DR (strong expression) and CD45 on the cell surface.

phenotypes.[20,21,28] Examples of LAIP's in AML samples at diagnosis are shown in Figure 4.

Due to their immunophenotypic heterogeneity, AML cells usually spread across many areas in each dot plot instead of forming a tight cluster, typically seen in acute lymphoblastic leukemia (ALL). Therefore, with any given marker combination, only a fraction of cells may appear to be phenotypically abnormal and consequently more than one subpopulation can be distinguished in the majority of AML.[20,29] In most patients (>75%) two or more subpopulations co-exist.

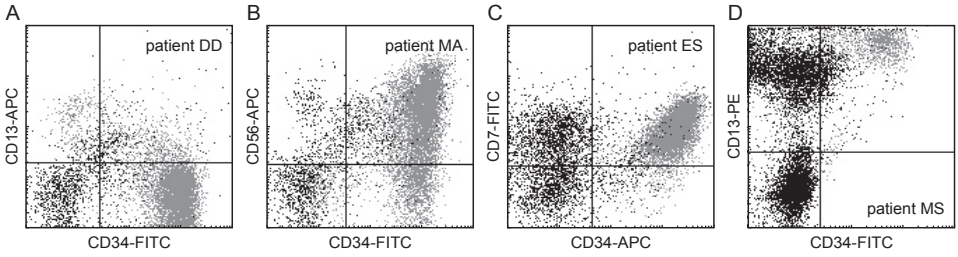


Figure 4. Flow cytometric examples of aberrant or unusual antigen expression patterns in four AML patients at diagnosis.
(A) Lack of CD13 expression on $CD34^+$ myeloid blast cells.
(B) Asynchronous expression of the more mature marker CD56 on $CD34^+$ myeloid blast cells.
(C) Cross-lineage expression of the T-lineage marker CD7 on $CD34^+$ myeloid blast cells.
(D) Over-expression of CD13 on the $CD34^+$ myeloid blast cells.

Applications of immunophenotyping in AML

The first stratification step in recent therapeutic protocols is the discrimination of either AML, ALL and acute undifferentiated leukemia (AUL). For proper lineage assessment of the leukemic cell population immunophenotyping is of utmost importance. Although morphologic evaluation completed with cytochemical stainings can identify several subtypes of AML, for the diagnosis of the AML-M0 (characterized by negative cytochemical reaction for myeloperoxidase at the light microscopic level, but by the expression of one or more myeloid markers) and the AML-M7 (characterized by the expression of platelet antigens such as CD41, CD42 and CD61) subtypes, immunophenotyping is the most powerful and rapid method (Table 1).[16,30]

In addition to classification into differentiation-based subtypes, detailed flow cytometric studies can define complex antigenic profiles that are associated with specific molecular defects.[31] Blast cells from an AML with t(8;21) show higher levels of CD34 and HLA-DR, lower CD33 expression and are more frequently positive for CD19 and CD56 surface markers when compared to t(8;21) negative AML.[32,33] Therefore, this highly predictive expression pattern ($CD34^+/CD19^+/CD56^+$) could serve as a screening criteria for the t(8;21). Immunophenotyping may also be of great value for a quick screening of acute promyelocytic leukemia (APL) with *PML-RARA* gene rearrangements. The presence of a heterogeneous expression of CD13, the existence of a single major blast cell population, negativity of HLA-DR, and a characteristic CD34/CD15 differentiation pattern show a high sensitivity and specificity for the prediction of *PML-RARA* gene rearrangements.[34] AML-M4 with eosinophilia and inv(16) exhibit a specific $CD2^+$ immunophenotype,[35] and also the AML-M3 variant often co-expresses CD2.[17,36]

A phenotypic characterisation of AML is essential if antibody targeted therapy is considered as a treatment option.[37] In > 85% of AML cases CD33 is expressed on the cell surface of the clonogenic leukemia progenitor cells. Treatment with a calicheamicin-conjugated anti-CD33 monoclonal antibody (gemtuzumab ozogamicin, Mylotarg®) has demonstrated substantial efficacy in patients with a $CD33^+$ AML.[38,39] Recent reports provide a basis for the development of other antibody targeted therapies in AML, including CD44-targeted therapy and CLL1-targeted therapy.[40-42]

Finally, immunophenotyping of AML can be used to study the therapy efficacy by monitoring bone marrow and peripheral blood samples for the occurrence of minimal residual disease (MRD).[43-46]

Immunophenotyping in CML

CML is a bi- or triphasic disease with an indolent chronic phase (CP), followed by one or both aggressive transformed stages, i.e. the accelerated phase (AP) and the blast crisis (BC). Flow cytometric immunophenotyping for CML patients in CP is of limited value. However, for patients in AP and especially for patients presenting in BC, flow cytometric immunophenotyping can help to identify the cell-lineage of

the blast cells. In about 70% of cases, the blast lineage is myeloid, while in 20-30% of patients, the BC is due to proliferation of lymphoblasts.[47-49] Rarely, patients have separate populations of myeloid and lymphoid lineage simultaneously. Additionally, identification of LAIP's in the blast cells can be used to monitor MRD.

1.2 MOLECULAR GENETIC ABNORMALITIES IN MYELOID LEUKEMIA

Currently, specific chromosome aberrations with fusion genes, mutated genes or aberrantly expressed genes are found in more than 70% of AML and more than 95% of CML. Major specific molecular markers in AML and CML are summarized in Table 2.

Chromosome aberrations with fusion genes in AML

The most common reciprocal rearrangements in AML in Northwest Europe include t(8;21)(q22;q22) and inv16(p13q22)/t(16;16)(p13;q22), giving rise to the fusion genes and fusion transcripts *AML1-ETO* and *CBFB-MYH11*, respectively (Table 2). AML1 and CBFβ are interacting proteins forming the core binding factor transcription complex which is essential for normal hematopoiesis.[50] Disruptions of the AML1/CBFβ complex is frequently implicated in leukemogenesis. *AML1-ETO* and *CBFB-MYH11* fusion transcripts occur in about 10-25% of all AML (dependent on age) and are associated with a favorable outcome.[51-53] *AML1-ETO* fusion gene transcripts are predominantly found in AML-M2.

The presence of *CBFB-MYH11* fusion genes strongly correlates with AML-M4Eo. Depending on distinct breakpoint locations, ten types of *CBFB-MYH11* fusion transcripts (A-J) are described.[54,55] More than 85% of *CBFB-MYH11* positive AML have a type A transcript; type D and E transcripts represent each nearly 5%, whereas all other types occur in sporadic cases.

APL or AML-M3 accounts for 2-5% of *de novo* AML (higher incidences are reported in Southern Europe)[56] and is associated with a favorable prognosis. APL is characterized by a chromosomal rearrangement involving the retinoic acid receptor alpha (*RARA*) gene that plays an important role in modulating myeloid differentiation. More than 95% of APL cases have a t(15;17)(q22;q21) with a *PML-RARA* fusion gene. While *RARA* breakpoints always occur within a ~15 kb fragment of intron 2, three breakpoint regions in the *PML* locus are involved in the t(15;17): intron 6 (bcr1; 55% of cases), exon 6 (bcr2; 5%) and intron 3 (bcr3; 40%).[57] Other fusion partners for *RARA* are *PLZF*, *NPM*, *NuMA*, and *STAT5b* as a result of t(11;17)(q23;q21), t(5;17)(q35;q21), t(11;17)(q13;q21), and t(17;17), respectively, but their frequency is low.[58]

Chromosomal translocations involving the mixed lineage leukemia (*MLL*) gene (also called *ALL-1*, *HRX*, or *TRX1*) on chromosome band 11q23 are not lineage

Table 2. Specific molecular markers in AML and CML.

Disease category and aberration	Frequency	
	Children	Adults
AML		
Aberrations with fusion genes		
Common recurrent abnormalities		
t(8;21)(q22;q22) with *AML1-ETO*	10-15%	6-8% [a]
t(15;17)(q23;q21) with *PML-RARA* [b]	8-10%	2-5% [a]
inv(16)(p13q22) and t(16;16)(p13;q22) with *CBFB-MYH11*	5-8%	5-7% [a]
11q23 aberrations with a *MLL* fusion[c]	15-20%	3-7%
Rare recurrent abnormalities		
t(6;9)(p23;q34) with *DEK-CAN*	~1%	
t(8;16)(p11;p13) with *MOZ-CBP*	~1%	
t(3;21)(q26;q22) with *AML1-EVI1*	~1%	
t(16;21)(p11;q22) with *FUS-ERG*	~1%	
Mutations of genes		
FLT3 mutations		
internal tandem duplications (ITD)	5-25%[d]	20-25% [e]
"activation loop" mutations[f]	ND	6-7%
RAS mutations	15-30%	
AML1/*RUNX1* mutations	ND	8%
CEBPA mutations	6%	8-11%
NPM1 mutations	6.5% [g]	35% [g]
Abnormal expression of genes		
WT1 overexpression	85-100%	85-100%
PRAME overexpression	60-65% [h]	35-50%
Ig/TCR gene rearrangements	5-10%	5-10%
CML		
Aberrations with fusion genes		
t(9;22)(q34;q11) with *BCR-ABL* (M-bcr breakpoint)	>95%	
t(9;22)(q34;q11) with *BCR-ABL* (m-bcr breakpoint)	2-3% [i]	
t(9;22)(q34;q11) with *BCR-ABL* (*μ*-bcr breakpoint)	<2%	

[a] Mainly concerns adult patients <60 years old. In patients >60 years old, the total frequency of *AML1-ETO*, *CBFB-MYH11* and *PML-RARA* fusion genes is <10%.[53]

[b] In Southern European regions, the frequency of t(15;17) with *PML-RARA* is essentially higher than in Northern European countries.[56]

[c] Seventy-one different partner chromosome regions have been detected, of which 51 partner genes are identified.[63-65]

[d] The occurrence of *FLT3*-ITD is age dependent: <1% in infant AML, 5% in children 1-10 years old and up to 19% in children 10-18 years old.[97]

[e] The frequency of *FLT3* mutations is lower in secondary AML (16%) and therapy-related AML (5-12%).[82,83] The frequency of *FLT3*-ITD in the elderly patients is higher.[101]

[f] Asp835Tyr is the most common substitution.

[g] Percentages in primary AML (FAB non-M3 subtypes).[115] There is an age dependent distribution: higher probabilities of *NPM1* mutations in older children.[116]

[h] Data from a limited number of patients (50 children with AML).[123]

[i] Co-expression of p190 is seen in >90% of CML with a M-bcr breakpoint.

Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; *FLT3*, Fms-like tyrosine kinase 3; *WT1*, Wilms tumor; *PRAME*, preferentially expressed antigen of melanoma; *NPM*1, nucleophosmin; Ig, immunoglobuline; TCR, T-cell receptor; BCR, breakpoint cluster region; ND, no data yet available or analyzed in limited patient series.

specific: they can be detected in AML, ALL, and mixed lineage acute leukemia. In ~60% of infant AML, *MLL* gene aberrations are found.[59,60] They are also frequently seen in childhood and adult patients with *de novo* AML[61] or secondary AML following therapy containing topoisomerase II inhibitors.[62] AML with *MLL* rearrangements is frequently associated with M4/M5 morphology. At present, 71 different partner chromosome regions have been found, of which at least 51 partner genes are identified.[63-66] The t(6;11)(q27;q23), t(9;11)(p22;q23), t(10;11)(p12;q23) and t(11;19)(q23;p13.3), resulting in *MLL-AF6*, *MLL-AF9*, *MLL-AF10*, and *MLL-ENL* fusion genes respectively, are the most common translocations in AML.[67-70] Regardless their association with other high-risk factors at presentation, *MLL* rearrangements are strongly predictive of poor clinical outcome. Although some reports have suggested that adult and childhood AML patients with t(9;11) carry a better prognosis,[71-73] this is not confirmed by others.[74,75]

Finally, several recurrent translocations are found at low frequency (≤ 1%) in AML (Table 2). The t(8;16)(p11;p13) results in a fusion of the CREB-binding protein gene (*CBP*) and the *MOZ* gene. It is a well described entity in both *de novo* and treatment-related AML,[76,77] associated with a young age at diagnosis, M4 or M5 morphology, erythrophagocytosis, coagulopathy, and poor outcome.[74] AML with t(6;9)(p23;q34) gives rise to the *DEK-CAN* fusion gene and fusion transcript. It is classified mostly as M2 or M4, and might show bone marrow basophilia. Patients with this type of leukemia are usually quite young[78] and poorly respond to therapy. Other rare genotypic aberrations in AML are t(3;21)(q26;q22), and t(16;21)(p11;q22) with the molecular targets *AML1-EVI1*, and *FUS-ERG* respectively.[79,80]

Gene mutations in AML

FLT3 mutations are the most common genetic abnormalities in AML: they involve either an internal tandem duplication (ITD) in the juxtamembrane domain-coding sequence or a point mutation in the activation loop domain.[81,82] The most common form of *FLT3* mutation is an ITD in exon 14 and 15 (previously known as exon 11 and exon 12), which occurs in *de novo* AML (~25%), secondary AML (~16%) and therapy-related AML (5-12%).[82,83] *FLT3*-ITD mutations can be of variable length -varying from 3 to up to 400 base pairs-, but they always consist of a multiplicity of three nucleotides, thereby retaining the reading frame.[84] In about 30% of *FLT3*-ITD, extra nucleotides are randomly inserted between the duplicated stretches. *FLT3*-ITDs lead to ligand-independent autophosphorylation of the receptor, resulting in proliferation and inhibition of apoptosis. Point mutations of *FLT3* in exon 20 (previously known as exon 17) occur in about 7% of AML. They predominantly involve the substitution of aspartic acid to tyrosine at codon 835.[85,86] Other less frequent substitutions include Asp835Val, Asp835His, Asp835Glu, Asp835Ala, and Asp835Asn. Mutations in codons surrounding codon 835 (e.g. codons 836, 840, 841, and 842) are reported as well.[87-90] Similar to ITDs, point mutations maintain the same open reading frame and result in a constitutively activated FLT3 protein. Some patients have both an

FLT3-ITD and a *FLT3* point mutation, however, most patients have only one type of *FLT3* mutation.[87,91] *FLT3*-ITDs are significantly associated with hyperleucocytosis and a high bone marrow blast count. The vast majority of patients have a normal karyotype.[82,92] However, an increased incidence (20-45%) of *FLT3* aberrations in patients with APL, especially the M3 variant[82,87,93] has been reported and *FLT3*-ITDs has been observed in 90% of patients with a *DEK-CAN* fusion gene.[87] *FLT3*-ITD is shown to be an indicator for a bad prognosis both in adults and children.[92,94-99] Also Asp835 mutations may confer poor prognosis in AML, although their clinical significance needs to be confirmed.[85,87] In APL patients, all studies to date failed to show an impact of *FLT3* aberrations on relapse and disease free survival.[93,100]

Mutations of the *N-* and *K-RAS* genes occur in 15-30% of AML,[102,103] with *N-RAS* mutations being the most common. The mutations usually involve single amino acid substitutions in codons 12, 13 or 61. Most *RAS* mutations occur as a single mutation in *N-RAS* or *K-RAS*, but two *RAS* mutations can coexist.[102,104,105] Reports addressing the clinical significance of *RAS* mutations in AML are inconclusive: some studies demonstrate a beneficial effect,[104,106] while others reached a different conclusion, e.g. lower complete remission in patients with *RAS* mutations.[107] To date, the prognostic value of *RAS* mutations seems to be of minor relevance compared to age or karyotype. *RAS* and *FLT3* mutations occur independently.[101,107]

Mutations of *CEBPA*, encoding the CCAAT/enhancer-binding protein-alpha, occur in ~6% of childhood AML and ~10% of adult AML.[108,109] They are highly associated with M1, M2 or M4 morphology and almost exclusively reported in patients belonging to the intermediate cytogenetic risk-group. The presence of *CEBPA* mutations is identified as a good prognosis factor for outcome in adults,[109,110] while the prognostic relevance in pediatric AML requires further investigation.

The overall frequency of *AML1* (also called *RUNX1)* point mutations in AML is ~8%.[111] *AML1* mutations are predominantly found in AML-M0 (16-27%);[112,113] especially biallelic mutations are tightly associated with AML-M0, while mono-allelic *AML1* mutations in AML-M0 are frequently observed in combination with *FLT3* mutations.[112] Most mutations (81%) are located in the DNA binding domain encoded by exons 3-5.[114]

Recently, *NPM1* (nucleophosmin) mutations in exon 12, resulting in an aberrant cytoplasmic localization of the NPM1 protein, are found in 35% of adult and in 6.5% of childhood patients with primary non-M3 AML.[115,116] *NPM1*-mutated AMLs show frequently a (myelo-)monocytic morphology (M4/M5) and have a lack of the hematopoietic stem cell markers CD34 and CD133. The *NPM1*-mutations are strongly associated with a normal karyotype and show a high frequency of *FLT*3-ITDs. Several studies showed that *NPM1*-mutations without *FLT3*-ITDs in AML with normal karyotype predict a favourable outcome.[117-119] The stability of *NPM*1 mutations between diagnosis and relapse is reported in a small patient group, suggesting that *NPM1* mutations might be useful to monitor MRD.[120]

Abnormal expression of genes in AML

In AML, abnormal expression of genes that are not involved in chromosomal translocations, include overexpression of the *WT1* gene (Wilms tumor) and overexpression of the *PRAME* gene (preferentially expressed antigen of melanoma). High levels of *WT1* can be found in the majority of AML patients at diagnosis;[121] the prognostic impact is however still uncertain. Overexpression of *PRAME* in AML is reported only in small series: high levels op *PRAME* are found in 35% of adult AML and 62% of childhood AML.[122,123] It is associated mainly with AML subtypes carrying a relatively favorable prognosis: AML with t(8;21), and APL with t(15;17).

Chromosome aberrations with fusion genes in CML

The hallmark of CML is the Philadelphia chromosome which results from a reciprocal translocation t(9;22)(q34;q11) with formation of a *BCR-ABL* fusion gene (Table 2) and hybrid protein.[124,125] The latter has a constitutively active tyrosine kinase function which induces aberrant oncogenic signaling.[126] Breakpoints in the *ABL* gene almost exclusively occur in a ~200 kb breakpoint region upstream of exon a2, while breakpoints in *BCR* are clustered in three well defined breakpoint-cluster regions (Figure 5).[127,128] The major breakpoint cluster region (M-bcr) is a ~2.9 kb chromosomal region between exon 13 and 15. It is detected in >95% of CML and leads to the formation of two types of mRNA molecules (e13a2 or e14a2, formerly assigned as b2a2 and b3a2), both encoding a $p210^{BCR-ABL}$ fusion protein. Secondly, the rarely occurring (2-3% of cases) minor breakpoint cluster region (m-bcr), spanning a ~55 kb intronic sequence between the two alternative exons 1 and 2, results in an e1a2 fusion gene transcript that encodes the $p190^{BCR-ABL}$ fusion protein. Thirdly, the micro-breakpoint cluster region (μ-bcr), a ~1 kb sequence located in intron 19 of the *BCR* gene, results in an e19a2 fusion gene transcript. This e19a2 fusion transcript encodes a $p230^{BCR-ABL}$ fusion protein which is mainly found in the rare neutrophilic variant of CML.[129,130] Table 3 shows the major molecular variants of *BCR-ABL* and its associated clinico-pathological features.[131-133]

Atypical *BCR-ABL* transcripts having junctions other than e13a2, e14a2, e1a2 or e19a2, are found in 1-2% of CML patients. They can be assigned to 4 groups according to common features of the *BCR* and *ABL* breakpoints within the fusion gene: 1) fusion genes in which *BCR* breakpoints are located outside the three recognized breakpoint cluster regions,[134,135] 2) fusion genes in which the breakpoint occurs within exons,[136] 3) fusions in which *ABL* breakpoints are located downstream of exon a2 giving rise to a hybrid mRNA with an a3 junction,[137] and 4) transcripts that contain 'bizarre' insertions or intervening sequences between *BCR* and *ABL*.[138,139]

Table 3. Major molecular variants of *BCR-ABL* and associated clinico-pathological features.

BCR breakpoint region	*Fusion transcript* [a]	*BCR-ABL fusion protein*	*Disease association*
M-bcr	e13a2 (b2a2) and/or e14a2 (b3a2)	$p210^{BCR-ABL}$	>95% of typical CML[b]
			10-20% of Ph+ childhood precursor-B-ALL
			25-45% of Ph+ adult precursor-B-ALL
			<1% of T-ALL
			<1% of AML
			27% of normal individuals [c]
m-bcr	e1a2	$p190^{BCR-ABL}$ [d]	2-3% atypical CML, often with monocytosis or dysplastic features
			>90% of CML with a classical *M-bcr* breakpoint [e]
			80-90% of Ph+ childhood precursor-B-ALL
			70-75% of Ph+ adult precursor-B-ALL
			<1% of AML (associated with M4 or M5)
			majority (69%) of normal individuals [c]
µ-bcr	e19a2	$p230^{BCR-ABL}$	neutrophilic variant of CML
			CML with marked thrombocytosis
			<1% of AML

[a] All fusion transcripts also can exist as splice variants involving exon a3 (see Figure 5).
[b] Approximately 20% of CML patients have both e13a2 and e14a2 transcripts due to alternative splicing.[131]
[c] Only detected with PCR-assays reaching sensitivities of 10^{-8} to 10^{-9}.[140,141]
[d] Also referred to as $p185^{BCR-ABL}$.
[e] e1a2 transcripts in patients with *M-bcr* breaks are most likely the result of alternative splicing of e13a2 or e14a2 transcripts.[142,143]

Abbreviations: BCR, breakpoint cluster region; Ph, Philadelphia chromosome; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia.

1.3 IMMUNOGENOTYPIC CHARACTERISTICS OF MYELOID LEUKEMIA

Surface membrane-bound immunoglobuline (Ig) molecules and T-cell receptor (TCR) molecules represent the antigen-specific receptors of mature B and T lymphocytes, respectively. Although the number of different gene segments in the Ig and TCR encoding genes is relatively limited (~ 470), an enormous diversity

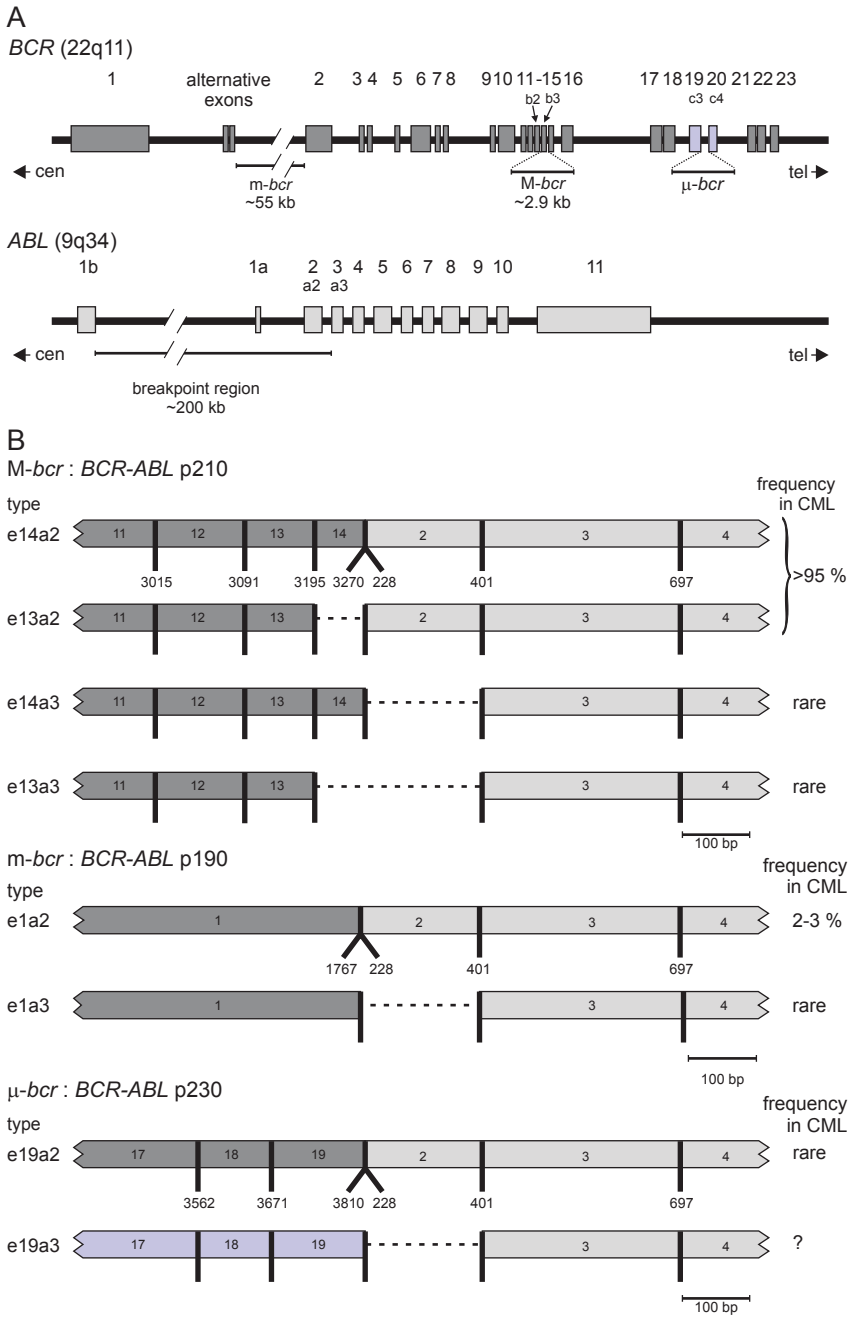

A
BCR (22q11)
1
alternative exons
2
3 4 5 6 7 8
9 10 11-15 16
b2 b3
17 18 19 20 21 22 23
c3 c4
cen
m-bcr ~55 kb
M-bcr ~2.9 kb
μ-bcr
tel
ABL (9q34)
1b
1a
2 3 4 5 6 7 8 9 10
a2 a3
11
cen
breakpoint region ~200 kb
tel
B
M-bcr : BCR-ABL p210
type
frequency in CML
e14a2
e13a2
e14a3
e13a3
3015 3091 3195 3270 228 401 697
>95 %
rare
rare
100 bp
m-bcr : BCR-ABL p190
type
frequency in CML
e1a2
e1a3
1767 228 401 697
2-3 %
rare
100 bp
μ-bcr : BCR-ABL p230
type
frequency in CML
e19a2
e19a3
3562 3671 3810 228 401 697
rare
?
100 bp

Figure 5.
A) Schematic diagram of the exon/intron structure of the *BCR* and *ABL* genes, involved in t(9;22)(q34;q11). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated. The old nomenclature of the *BCR* and *ABL* exons is indicated.
B) Schematic diagram of the three *BCR-ABL* transcripts (p190, p210 and p230) found in CML. The numbers under the fusion gene transcript refer to the first (5') nucleotide of the involved gene, except when the last (3') nucleotide of the upstream gene is indicated. The e19a3 junction is included as a theoretical possibility, however, no cases have been reported yet.

of the antigen-specific receptors is possible owing to the fact that combinations of gene segments are made, which are different in each lymphocyte and lymphocyte clone. Clonal Ig and TCR gene rearrangements are found in virtually all ALL patients, and they can also be seen in a subset of AML patients.

Ig and TCR gene rearrangements

During early B- and T-cell differentiation, germline V (variable), D (diversity), and J (joining) gene segments of the Ig and TCR genes rearrange and each lymphocyte thereby obtains a particular combination of V-(D-)J segments. In *IGH*, *TCRB*, and *TCRD* rearrangments one of the J gene segments is joined to one of the D gene segments, and subsequently a V to D-J joining occurs. In *IGK*, *IGL*, *TCRA*, and *TCRG* rearrangements, one of the J gene segments is directly joined to one of the V gene segments, resulting in a V-J joining. At the coupling sites of the gene segments, deletions and random insertions of nucleotides occur. An example of an *IGH* gene rearrangement is illustrated in Figure 6.

This lymphoid-specific process is mediated via the V(D)J recombinase complex, including the recombination activating gene products (*RAG1* and *RAG2*),[144,145] which recognize specific recombination signals sequences (RSS). The RSS consists of a palindromic heptamer and nonamer sequence, separated by spacer regions of 12 or 23 base pairs. The RSS borders the 3' side of V gene segments, both sides of D gene segments and the 5' side of J gene segments.[146] TdT plays an important role during this Ig and TCR gene rearrangements by random insertion of nucleotides at the junction side of the V-(D-)J gene segments.[147]

Although Ig and TCR molecules are only expressed by B and T lymphocytes, respectively, rearrangements of *IG* and *TCR* genes are not lineage restricted.[148] In precursor-B-ALL high frequencies of *TCR* gene rearrangements are found and cross-lineage *IG* gene rearrangements occur at low frequency in T-ALL.[149,150]

Also AML show cross-lineage Ig and TCR gene rearrangements in approximately 10% of cases (Table 2). *IGH* and *IGK* gene rearrangements are found in 10% and 3% of cases, respectively.[151,152] The *IGH* gene rearrangements mainly concern incomplete DH-JH joinings (>90%) with frequent usage of the more downstream DH gene segments (>90%) and the JH4 and JH6 gene segments.[152] So far only one AML case has been reported having a rearranged Ig lambda locus.[153] *TCRD, TCRB*, and *TCRG* gene rearrangements are found in 9%, 7%, and 5% of AML patients,[151,152,154]

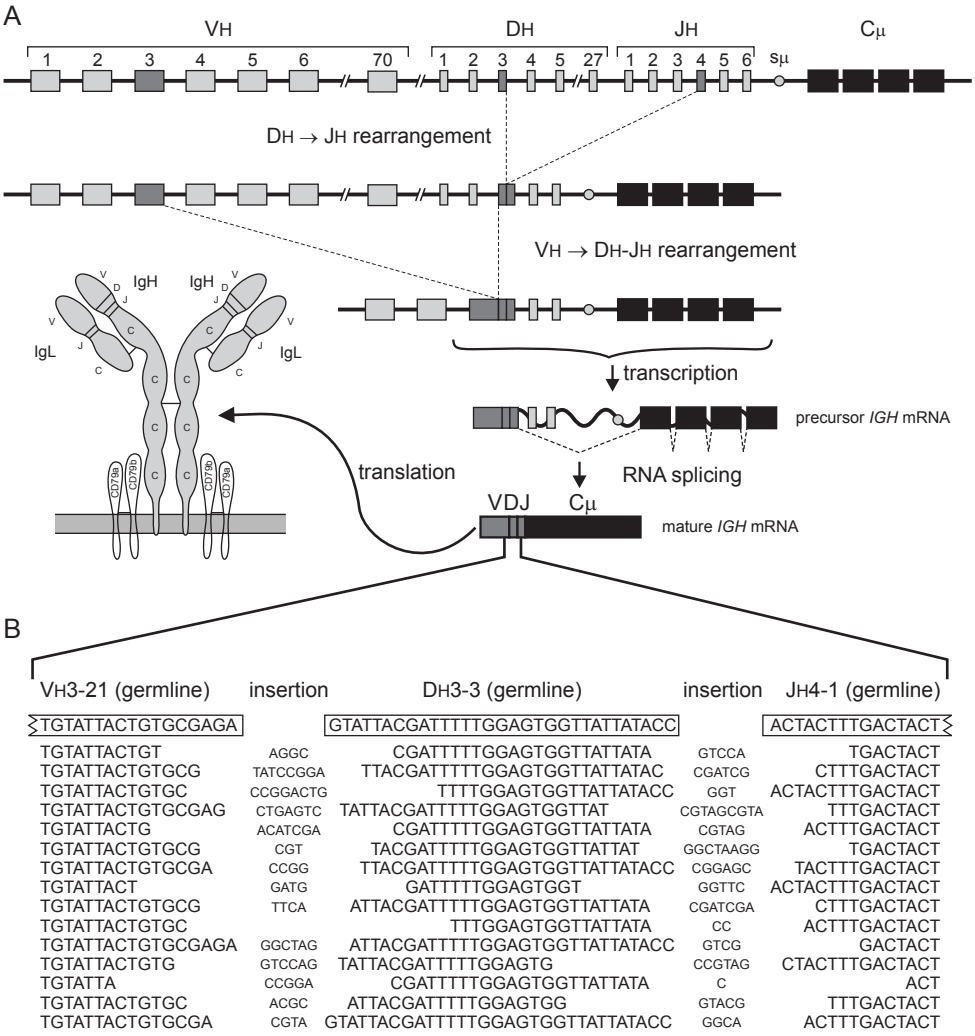


Figure 6.

A. Schematic diagram of human *IGH* gene rearrangement. In this example D_H3 is first joined to J_H4, followed by V_H3 to D_H3-J_H4, thereby deleting all intervening sequences. This rearranged gene complex can be transcribed into precursor mRNA, which will be transformed into mature mRNA by splicing out all non-coding intervening sequences. Finally, the transcript will be translated into an IgH chain.

B. Schematic diagram of the junctional regions of the joined V_H3, D_H3, and J_H4 gene segments. The at random insertion and deletion of nucleotides at the junction sites of V, D, and J gene segments make the junctional regions into 'fingerprint-like' sequences, which are probably different in each lymphocyte. Germline and inserted nucleotides are indicated by capital letters and small letters, respectively.

respectively. This frequencies are in concordance with the physiological hierarchy of TCR gene rearrangements during T cell differentiation. Studies on AML-M0 cases show higher incidences (22%) of *IGH/TCR* gene rearrangements.[113] However, no prognostic significance can be linked to the presence of clonal Ig/TCR gene rearrangements in AML.[155-157] The occurrence of Ig/TCR gene rearrangements in AML and its immunophenotypic characteristics has been investigated. Some studies reported significant associations between positivity for B cell-associated markers and the presence of Ig/TCR gene rearrangements,[152] others correlated the expression of T cell markers or TdT with the occurrence Ig/TCR gene rearrangements.[153,154,158,159]

Small series report on the occurrence of Ig/TCR gene rearrangements in CML and show close similarities between the genotypic features of B-lymphoid BC CML and precursor-B-ALL.[160,161] Also in myeloid lineage BC CML, Ig/TCR gene rearrangements are reported.[162] Finally, in CP CML *IGH* gene rearrangements can be detected from seven months prior to the development of lymphoid BC.[163,164]

2. MODERN THERAPY OF MYELOID LEUKEMIA

2.1 STANDARD TREATMENT MODALITIES IN MYELOID LEUKEMIA

Acute myeloid leukemia

The initial treatment of newly diagnosed AML consists of a remission induction regimen to achieve two goals: firstly, the reduction of the malignant BM blast percentage to ≤ 5%, and/or the elimination of overt extramedullary disease, and secondly, the restoration of normal hematopoiesis. Having achieved this, the therapeutic goal is to prevent relapse. Strategies to improve leukemia-free and overall survival, include consolidation therapy, maintenance therapy or stem cell transplantation (SCT).

Anthracyclines and cytarabine are cornerstones of modern AML therapy. Pediatric AML induction is based on the standard “7+3” induction regimen (i.e. 7 days cytarabine and 3 days anthracyclines), and the adding of a third agent such as 6-thioguanine or etoposide.[165-167] These schemes induce a remission in approximately 85% of pediatric patients. In adult AML, modern remission induction schedules are also based on anthracyclines given at 3 days and cytarabine given over 7 to 10 days, possible combined with a third drug.[168] These schedules induce complete remission in about 75-80% of younger patients (<60 years) and 50-55% of older patients.[169]

Post-remission therapies are based on high-dose cytarabine with or without autologous or allogeneic SCT.[170,171] Several pediatric cooperative groups addressing

the role of autologous SCT in post-remission therapy, failed to show a benefit of autologous SCT over chemotherapy alone.[172] The optimal number of cycles of intensification chemotherapy in children is not known[166,171,173,174] nor is the role of ALL-like maintenance therapy.[175] In adults, post-remission chemotherapy is nowadays limited in duration (2 or 3 consolidation courses), but made more intensive.[176,177]

In contrast to children and younger adults, the outcome results of elderly patients with AML has not improved substantially during the last decades. Older adults with AML have a dismal long-term outcome with a 5-year survival of less than 5 to 8%.[178,179] Treatment in the elderly AML patients is more difficult by their limited ability to tolerate intensive chemotherapy. No standard therapy is available for patients aged ≥ 80 years of age. For patients aged 61-79 years, induction therapy might be similar to the standard therapy in younger AML patients, however, for post-remission therapy so far no standard strategy exists.

Eight to 10% of younger AML patients have resistant disease and 10% will die due to treatment toxicities.[180] The overall relapse risk for younger AML patients achieving complete remission is 45-50%, but primarily depends on important risk factors such as cytogenetics, age and response of the BM to the first course of chemotherapy.[181,182]

In patients with refractory or relapsed disease, reinduction regimens typically use high-dose cytarabine, as well as mitoxantrone, etoposide, or fludarabine.[183,184] For consolidation, patients preferably undergo allogeneic SCT with either a related or unrelated donor.[185] Despite the risks associated with allogeneic SCT, between 20-50% of patients can be salvaged.[186,187] The salvage rates are significantly affected by the duration of the first complete remission (CR1), with shorter CR1 (<6 months or while on therapy) being associated with lower salvages rates.[188]

It is well accepted to consider APL as a separate entity, requiring a specific therapeutic approach involving all-*trans*-retinoic acid (ATRA). This is discussed in paragraph 2.2.

The trend of dose intensification has largely explored the therapeutic potential of conventional drugs with toxicities precluding further dose escalation. Therefore, the focus is shifting now to the development of less toxic alternative strategies selectively targeting the leukemic cells.

Chronic myeloid leukemia

The treatment goal in CML is the elimination of the Philadelphia (Ph) positive clone and the restoration of Ph-negative hematopoiesis. Treatment decisions in CML patients primarily are based on the patient's age and the phase of the disease.

The conventional chemotherapeutic agents busulfan and hydroxyurea (HU) control the elevated white blood count and the disease-related symptoms in a majority of CML, without a demonstrable effect on the natural history of the

disease: they fail to induce a cytogenetic remission, nor do they delay the onset of the acceleration phase (AP) or blast crisis (BC). Consequently, their effects are primarily palliative.[189]

In contrast, interferon (IFN) in monotherapy or in combination with subcutaneous Ara-C, can induce complete hematologic (22-87%) and major cytogenetic responses (10-46%) in patients with chronic phase (CP) CML.[190] In patients with advanced disease, IFN demonstrated less activity.[191] However, IFN is not curative and 10-25% of patients discontinue their therapy because of intolerance.[192,193]

To date allogeneic SCT is the only treatment modality with an established curative potential.[194-196] The most significant determinants of transplantation outcome are the patient's age, the phase and duration of the disease. Disease-free survival (DFS) with matched-related allogeneic SCT are 40-80% in CP phase, 15-40% in AC phase, and 5-20% in BC. In CP CML, patients <30-40 years have a DFS of 60-80%, while in patients >45 years of age a 5-year DFS of 25% is reported.[197] Unfortunately, allogeneic SCT is only an option for 25-40% of patients and it carries substantial risks of treatment related morbidity and mortality. The probability of SCT-related death ranges from 20-40%,[198] with a higher mortality risk for transplants from unrelated or non-HLA-matched donors. However, in younger patients outcomes may be similar in molecularly matched unrelated SCT compared to related SCT.[199,200] Nonablative preparative regimens (mini-transplants or reduced intensity transplants) have attempted to expand the indications of allogeneic SCT to older patients and to reduce transplant related mortality and complications. Early results demonstrate a reduced morbidity and mortality,[201] but a higher incidence of persistent or recurrent disease, which could be approached with post-SCT regimens such as donor lymphocyte infusions.[202,203] For patients without a donor, until recently IFN/Ara-C was considered the gold standard treatment, although elderly CML patients often were excluded or treated with IFN at reduced dosages due to the higher drug toxicity.

2.2 TARGETED TREATMENT MODALITIES

A problem with standard chemotherapeutical agents is their toxicity towards normal cells. Therefore, therapeutic strategies that deliver the cytotoxic agent preferentially to the tumor are developed. These targeted therapies include monoclonal antibodies, e.g. gemtuzumab ozogamicin, and agents targeting the molecular basis of the disease, e.g. all-*trans*-retinoic acid and tyrosine kinase inhibitors which will further be discussed. In addition, other agents such as farnesyltransferase and histone deacetylase inhibitors, proteasome inhibitors and antiangiogenesis agents have recently been tested.

All-*trans*-retinoic acid (ATRA)

APL cells harbor the t(15;17), resulting in the *PML-RARA* fusion gene transcript and protein, which is leukemogenic. APL cells are sensitive to all-*trans*-retinoic acid (ATRA) which binds to PML-RARA and inhibits its anti-differentiation transcriptional activity.[204, 205]

ATRA used as a single agent induces remission in the majority of patients with APL, however, disease recurrence uniformly occurs within 3 to 4 months without additional therapy.[204] Multiple trials have demonstrated a benefit for ATRA in combination with chemotherapy[206,207] with recent evidence suggesting that anthracyclines play a more important role than cytosine arabinoside.[208,209] ATRA may also have a role in maintenance therapy after successful consolidation, although studies are ongoing to evaluate if this prolongs event free survival, especially in patients with a high initial leukocyt count (≥ 10.000/μL).[206,210]

Patients who relapse after ATRA plus chemotherapy can be induced into a second remission with ATRA or arsenic trioxide (ATO). The likelihood of a second response is considerable, particularly if several months have passed since the last administration of this agent. After remission re-induction, intensive chemotherapy for consolidation is needed to produce durable molecular remissions. Administration of ATO in relapsed APL results in a remission induction rate of ~85% and a molecular remission of approximately 70%, particularly after two cycles of treatment.[211-213] APL patients in ≥ second molecular remission are usually further consolidated using SCT.[214]

The introduction of ATRA, has changed the clinical course of APL from an often fatal disease to one of the most curable subtypes of AML.[206,215,216] However, clinical ATRA-resistance in APL (reviewed by Gallagher[217]) may hamper the response rates. Primary resistance of APL to ATRA as a single agent or in combination with chemotherapy, is rare both in *de novo* APL and in APL at first relapse from previous chemotherapy or in cases initially refractory to chemotherapy. By contrast, secondary acquired ATRA resistance is very common especially in patients treated continuously with oral ATRA as a single agent. Acquired ATRA resistance has been linked to both systemic (e.g. increased catabolism) and APL cellular (e.g. decreased nuclear transport) elements. ATRA resistance is found in 10-30% of patients who relapse from a CR achieved and consolidated with ATRA-containing regimens.[217] Fortunately, ATRA resistant disease appears highly sensitive to ATO. Consequently, ATRA and ATO have drastically changed the clinical outcome in APL.

Gemtuzumab ozogamicin (GO)

Gemtuzumab ozogamicin (Mylotarg®, formerly know as CMA-676), is an immunoconjugate in which a humanized IgG_4 CD33 monoclonal antibody is chemically linked to N-acetyl gamma calicheamicin through an acid-labile linker. Calicheamicin is a highly potent antitumor antibiotic (belonging to the class of anthracyclines) that cleaves double-stranded DNA.[218] The molecular target of GO is

the surface CD33 antigen, which is expressed on early multilineage hematopoietic progenitors and myelo-monocytic precursors, but not on normal hematopoietic stem cells and nonhematopoietic tissues.[219,220] Eighty-five to 90% of AML are $CD33^+$ as defined by the presence of CD33 on more than 20-25% of the leukemic cells. GO binds CD33 with high affinity and the antibody-antigen complex is rapidly internalized, finally leading to cell death. Figure 7 is a schematic representation of the mechanism of action of GO.

In elderly $CD33^+$ AML patients in first relapse and who were not considered candidates for other cytotoxic chemotherapy, GO resulted in an overall second remission of 30%, when administered as a single agent at a dose of 9 mg/m^2 as two infusions 14 days apart.[221,222] Based on these results, GO received an US FDA approval for treatment of relapsed AML patients over 60 years of age.

Several groups have evaluated the potential of GO in different situations of AML. Reports from small patient series suggest activity of GO as a single agent[223,224] or in combination regimens[225,226] as front line therapy in the elderly. There is some clinical experience in children with relapsed or refractory AML treated with GO on compassionate use;[227,228] results demonstrated that GO has clinical activity in these children showing 40-55% responders with a blast reduction in the BM to ≤ 5%. However, complete remission required additional intensive therapy, such as allogeneic SCT. Recent studies suggested that GO as a single agent is effective for patients with molecularly relapsed APL,[229,230] while GO combined with all-*trans*-retinoic acid appears active in untreated APL.[231] A feasibility study in younger AML patients showed that the simultaneous administration of GO with intensive chemotherapy as first line treatment induced a remission in 91% of patients.[232]

Potential mechanisms of GO resistance and treatment failure include the escape by CD33-negative leukemic cells, drug efflux via permeability glycoprotein or multidrug resistance protein 1,[233,234] anti-apoptotic effects of Bcl-2 or Bcl-X_L, and high circulating CD33 antigen burden leading to compromised drug delivery to BM.[235]

Imatinib mesylate (IM)

The discovery of the *BCR-ABL*-mediated pathogenesis of CML provided the rationale for the design of inhibitory agents that target BCR-ABL kinase activity. In 1998, imatinib mesylate (Gleevec®, Glivec®, formerly STI571), a selective inhibitor of ABL and its derivative BCR-ABL tyrosine kinase, was introduced to the clinic for the treatment of CML.[236] Imatinib (IM) binds the kinase domain of BCR-ABL thereby inhibiting its activity and selectively eradicating the leukemic cells with a low toxicity profile. Figure 8 is a schematic representation of the mechanism of action of IM.

IM is effective as a single agent for the treatment of patients in all stages of CML, whether newly diagnosed or previously treated. However, most encouraging results are seen in CP CML.[237-241] The International Randomized Study of Interferon and STI571 (IRIS) showed superior outcomes for newly diagnosed

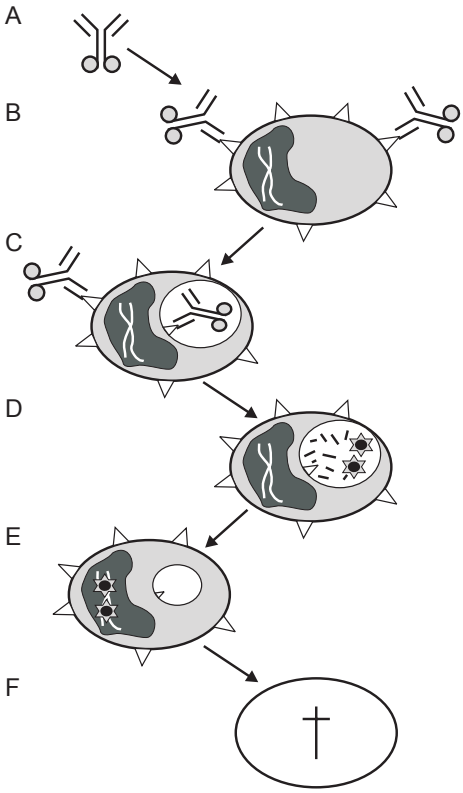


Figure 7. Mechanism of action of gemtuzumab ozogamicin (Mylotarg®).
(A) Gemtuzumab is a humanized CD33 murine monoclonal antibody linked to the antitumor antibiotic calicheamicin. After binding of gemtuzumab to surface CD33-antigens on normal and leukemic cells (B), the CD33-antigen/gemtuzumab complexes are rapidly internalized (C). Upon internalization of these complexes, newly produced CD33-antigens are rapidly expressed on the cell surface and subsequently can bind gemtuzumab (D). The rate of renewed CD33-antigen expression appears to be related to the degree of CD33 saturation and internalization, and to the activation status of the cell. The internalization is followed by hydrolysis of the acid-labile linker in the intracellular endosomes and lysosomes, the degradation of the antibody part of gemtuzumab, and finally by the activation of the calicheamicin γ1 derivate (D). The active calicheamicin enters and accumulates in the nucleus, binds to the minor groove of DNA and causes double-stranded DNA breaks (E). This results in the induction of cell death and apoptosis (F).

CP CML; complete hematologic and major cytogenetic response rates of 95% and 85% respectively at 18 months, and major molecular response rates of ≥3 log at 12 months in 39%.[240,241] An update at 30 months showed that for patients who

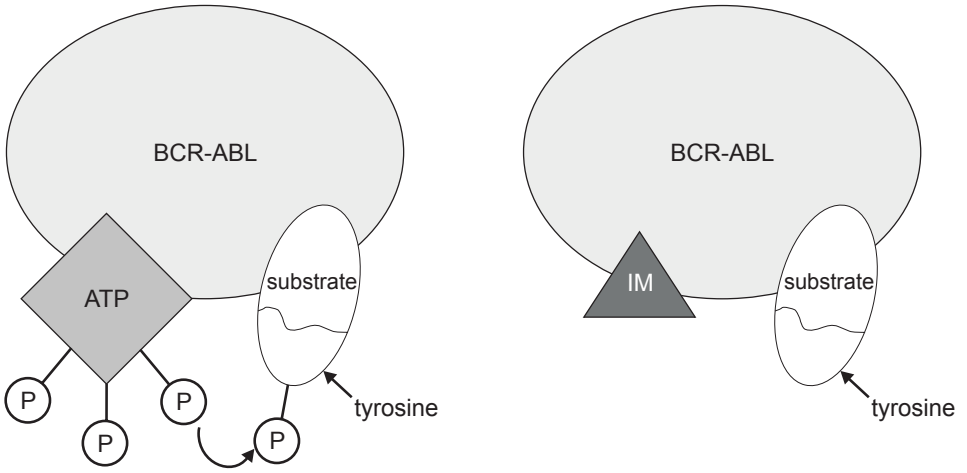


Figure 8. Mechanism of action of imatinib mesylate (Gleevec®).
Imatinib mesylate (IM) competes with adenosine triphosphate (ATP) for occupancy of its specific binding pocket within the kinase domain of the BCR-ABL protein. When the binding pocket is occupied by ATP, BCR-ABL can catalyse the phosphorylation of tyrosine residues on substrate proteins (left). IM is structural similar to ATP and can bind to the ATP-binding pocket within the BCR-ABL (right). Because IM lacks the essential phosphate groups that are normally provided by ATP, phosporylation of tyrosine residues on substrate proteins cannot take place. Substrate proteins with unphosphorylated tyrosine residues fail to adopt the necessary conformation for binding to effector molecules. Consequently, downstream activation of signal transduction pathways crucial for CML leukemogenesis are inhibited.

achieve a complete cytogenetic remission (CCyR) and a reduction in *BCR-ABL* transcript levels of ≥3 log at 12 months, the probability of remaining progression free was 100% compared to 93% for patients with < 3 log reduction in *BCR-ABL* transcript levels and 82% for patients who were not in CCyR at 12 months.[242] Data on long-term disease-free survival and overall survival are awaited as follow-up continues. Small studies also have shown a significant activity of IM against CML in relapse after allogeneic or autologous SCT.[243,244] Because IM monotherapy has shown to be significantly more effective and better tolerated than IFN/Ara-C, it has been approved as first-line treatment for patients with a new diagnosis of CML. The recommended starting dosages are 400 mg for CP CML and 600 mg for AP/BC CML.[245,246]

Although IM is unquestionably effective in the treatment of CML, some patients may ultimately relapse with resistant disease. A minority of patients in CP and a substantial proportion in advanced disease phases are either initially refractory to IM treatment or lose IM sensitivity over time and experience relapse.[247,248] Clinical resistance is primarily mediated by reactivation of BCR-ABL kinase activity within the leukemic cells by either point mutations within the kinase domain of *BCR-ABL*

or, to a lesser extend, by amplification of the *BCR-ABL* genomic locus.[249-251] Point mutations within the tyrosine kinase binding site can prevent IM from binding by interrupting critical contact points between IM and the protein, or by precluding access of IM to its binding site. Usually a single point mutation is detected, but occasional patients carry more than one *BCR-ABL* point mutation.[252,253] The incidence of *BCR-ABL* mutations increases with CML disease progression.[254,255] To date, approximately 30 different point mutations that encode for distinct single amino-acid substitutions in the BCR-ABL kinase domain have been identified in the CML cells from relapsed patients resistant to IM.[253] *In vitro* studies have demonstrated that some mutants (e.g. T315I) have 1 to 2 log higher 50% inhibitory concentration (IC_{50}) values for IM compared to wild-type BCR-ABL, whereas others have slightly higher IC_{50} values.[256,257] Another major IM resistance mutation which also enhances the activity of BCR-ABL kinase is E255K.[258]

Clinical management to avoid or overcome IM resistance might include dose escalation (depending on the type of the mutation), cessation or temporary interruption of IM therapy, and upfront or second-line combination therapy. Recently, second-generation molecular targeted therapies, including AMN107 and BMS354825, have been designed and tested in phase I trials for patients with IM resistant CML.[259-263] Both agents showed an increased potency with sensitivities against all *BCR-ABL* point mutations except for T315I mutation. Currently phase II studies are being started. Recently, a new non-ATP-competitive inhibitor of BCR-ABL showed *in vitro* activity against all IM resistant mutants, including T315I mutations.[264,265] Both the introduction of IM and the development of second-generation molecular targeted therapies represent a big step forward in the clinical management of CML patients.

3. MINIMAL RESIDUAL DISEASE DIAGNOSTICS FOR EVALUATION OF TREATMENT

Modern treatment protocols for AML induce a complete remission,[175,266,267] cytomorphologically defined as the presence of less than 5% blast cells in the bone marrow (BM), in a high proportion of leukemia patients (50-80%, depending on the age). However, many patients ultimately relapse, implying that current treatment protocols are not capable of killing all clonogenic malignant cells in these patients. To trace low frequencies of malignant cells, so called minimal residual disease (MRD), more sensitive techniques are required (Figure 9). MRD techniques should reach sensitivities of at least 10^{-3} (one leukemic cells in 1000 normal cells), but sensitivities of 10^{-4} to 10^{-6} are preferred.

Several large scale studies have shown that monitoring of MRD in malignant disease predicts clinical outcome. In both childhood and adult ALL, detection of MRD can be used to evaluate early treatment response and to improve stratification.[268-271]

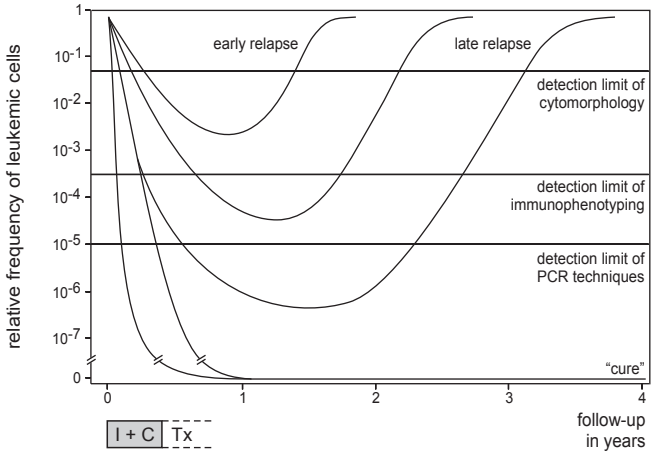


Figure 9.
Diagram of the relative frequencies of leukemic cells in peripheral blood or bone marrow of patients with acute myeloid leukemia, during and after therapy, and during development of relapse. The detection limit of cytomorphology, flow cytometric immunophenotyping and PCR techniques are indicated. I: induction, C: consolidation, Tx: transplantation.

In APL and CML, MRD information obtained at specific time points enables effective early treatment intervention.[272-274] Also in AML, different risk groups can be identified based on the MRD levels at specific therapeutic time points.[275-277] MRD information leads not only to the identification of patients at high risk of relapse, but may also be a first step towards reduction of treatment intensity in patients who might currently be over-treated.

3.1 TECHNIQUES FOR MRD DETECTION

During the past decades, several methods for MRD detection have been developed and evaluated, including conventional cytogenetics, fluorescent *in situ* hybridization, Southern blot analysis, immunophenotyping, and polymerase chain reaction (PCR). Most of these techniques are not suitable for clinical MRD detection due to limited sensitivity, limited specificity, or limited applicability.

At this moment, three quantitative techniques reaching sensitivities of 10^{-3} to 10^{-6} are widely used for detection of MRD in leukemia patients; (1) multiparameter flow cytometric immunophenotyping, based on the occurrence of leukemia-associated aberrant, unusual, or ectopic phenotypes, (2) PCR analysis of junctional

regions of rearranged Ig and/or TCR genes, and (3) PCR analysis of fusion gene transcripts associated with chromosome aberrations. In addition, PCR analysis of *FLT3* mutations and aberrantly expressed genes (e.g. *WT1*) have recently been evaluated as PCR MRD-markers in AML. The choice of the technique primarily depends on the applicability of the technique and the required sensitivity (Table 4). Table 5 summarizes the advantages and disadvantages of the different MRD techniques.

MRD detection by flow cytometric immunophenotyping

Acute leukemias can be regarded as malignant counterparts of cells in immature stages of hematopoiesis. Consequently, the presence of normal hematopoietic cells may limit the immunophenotypic detection of leukemic cells. Despite this inherent limitation, immunophenotypic MRD detection is still possible because leukemia associated phenotypes (LAIP's) can be identified in the vast majority of AML patients (>80%).[20,21,28]

It should be noted that the immunophenotype of the leukemic cells may be heterogeneous and that several subpopulations can be present at diagnosis.[29,278] As relapses can occur by a less represented subpopulation, all leukemic subpopulations should be monitored for reliable MRD analysis.[279]

A possible pitfall of immunophenotypic MRD detection in acute leukemia is the occurrence of immunophenotypic shifts during the course of the disease, which

Table 4. Sensitivity and applicability of MRD-targets in myeloid malignancies.

	PCR analysis				Immunophenotyping
	Ig/TCR gene rearrangements	*Fusion genes*	*FLT3-ITD*	*WT1*	*LAIP*
Sensitivity	10^{-4}-10^{-5}	10^{-4}-10^{-6}	10^{-4}-10^{-5}	10^{-3}-10^{-4}	10^{-3}-10^{-4}
Applicability					
AML children	5-10%	20-40%	5-25%	85-100%	75-85%
AML adults	5-10%	10-20%[a]	20-25%	85-100%	75-85%
APL	ND	>95%	20-45%	ND	>95%[c]
CML	ND	>95%	<2%	ND[b]	75-85% (myeloid BC) 60-98% (B-lymphoid BC) 90-95% (T-lymphoid BC)

[a] Mainly concerns adults <60 years of age. Lower frequencies are reported in elderly AML patients (>60 years of age).[50]

[b] *WT1* expression in blast crisis (BC) is higher than in chronic phase.

[c] APL with a *PML-RARA* gene rearrangement have a specific phenotypic pattern.[34]

ND: no data yet available or only analyzed in a limited number of patients.

Table 5. Advantages and disadvantages of MRD techniques.

MRD technique	Advantages	Disadvantages
Flow cytometric immunophenotyping	– single cell analysis – cell viability can be determined – information on normal cells – relatively easy and cheap – rapid (1-2 days) – relatively patient-specific – applicable for most patients	– immunophenotypic shift – presence of subpopulations in AML – background of normal cells – limited sensitivity (10^{-3} – 10^{-4})
PCR analysis		
▪ fusion genes (mRNA level)	– stable target (related to oncogenesis) – relatively easy – rapid (1-3 days) – no (or very low <10^{-6}) background in normal cells – suitable for monitoring uniform patient groups – sensitive (10^{-4} – 10^{-6})	– instability of RNA – not patient but tumor specific – risk of contamination – variable expression levels (may be affected by therapy) – useful in only a subset of AML patients (5-30%)
▪ fusion genes (DNA level)	– patient specific – low risk of contamination – high stability of DNA – stable target (related to oncogenesis) – no (or very low <10^{-6}) background in normal cells – suitable for monitoring uniform patient groups – sensitive (10^{-4} – 10^{-6})	– labor intensive identification of exact breakpoints, except for some translocations with small breakpoint regions, eg. *μ-bcr* rearrangements (e19a2 junction) – useful in only a subset of AML patients (5-30%)
▪ Ig/TCR gene rearrangements (DNA level)	– patient specific – low risk of contamination – DNA amount per cell is relatively constant – rapid during follow-up – high stability of DNA – sensitive (10^{-4} – 10^{-5})	– background of normal cells – high complexity – labor intensive and time consuming at diagnosis – relatively expensive – loss of target due to clonal evolution (ongoing/secondary rearrangements and oligoclonality) – useful in only a minority of AML patients (<10%)
▪ mutated genes: *FLT3*-ITD (DNA level)	– related to oncogenesis – patient specific – can be identified with limited set of primers – high stability of DNA – DNA amount per cell is relatively constant – no background in normal cells	– target may not be stable – useful in subset of patients only (10-25%)
▪ mutated genes: *FLT3*-ITD (mRNA level)	– related to oncogenesis – patient specific – no background in normal cells	– instability of RNA – target may not be stable – useful in subset of patients only (10-25%)
▪ aberrant expressed genes: *WT1* (mRNA level)	– easily identified – applicable for most patients	– instability of RNA – not tumor specific – background expression in normal cells

may concern up to 90% of childhood and adult AML.[279-283] These shifts include a gain or loss of myeloid antigens (CD13 and CD33), changes in expression of cross-lineage markers (CD2, CD7, CD19, and CD56) or changes in the progenitor-associated antigens (CD34 and CD117). However, at least one leukemia-specific marker combination is retained by the leukemic cells at relapse in virtually all AML patients.[280] This implies that preferably at least two marker combinations per patient should be used for immunophenotypic MRD monitoring. Some studies reported that AML tends to relapse with a less differentiated phenotype than observed at diagnosis; they suggested that antibody panels used for MRD monitoring should not only be restricted to the immunophenotypes detected at diagnosis, but also should include markers of lineage immaturity.[279,284]

MRD detection by PCR analysis of fusion genes associated with chromosome aberrations

Almost all CML and 20-30% of AML patients have characteristic tumor-specific chromosome aberrations with the formation of fusion genes. These fusion genes are generally monitored at the RNA level, while monitoring at the DNA level is only sporadically done.

At the RNA level, the fusion genes are transcribed into fusion gene transcripts, which are similar in individual patients despite distinct breakpoints at the DNA level. After reverse transcription into cDNA, the fusion gene transcripts are used as targets for quantitative MRD-PCR analysis. Primer and probe sets for real-time quantitative PCR (RQ-PCR) based detection of the most common fusion gene transcripts as well as control gene transcripts, have been designed.[285,286] Generally, sensitivities of at least 10^{-4} can be obtained using these RQ-PCR approaches.

Low levels of fusion gene transcripts can be present in healthy individuals; for instance, *BCR-ABL* e13a2/e14a2 and *BCR-ABL* e1a2 transcripts are found in 27% and 69% of normal individuals, respectively.[140,141] Although the prevalence of *BCR-ABL* carrying leukocytes in the blood of healthy individuals is extremely low, probably less than 1 to 10 per 10^8 leukocytes, the presence of these fusion gene transcripts may hamper MRD detection, if the sensitivity of the RQ-PCR assay is extremely high.

Because of the high sensitivity of PCR techniques, cross-contamination of RT-PCR products between patient samples can be a pitfall in RT-PCR-mediated MRD studies employing fusion gene transcripts. Such cross-contamination is difficult to recognize, since fusion-gene RT-PCR products are leukemia-specific but not patient-specific. Furthermore, RNA is rather instable and differential stability of fusion gene transcripts and control gene transcripts may result in unreliable MRD data.[287-289] An additional (theoretical) disadvantage of applying RNA targets is that the fusion gene transcription process might be affected by the cytotoxic treatment, potentially resulting in transcript levels that differ per treatment phase.

MRD-PCR analysis of chromosome aberrations with fusion genes can also

been performed at the DNA level. Advantages of this approach include the patient specificity of breakpoint fusions at the DNA level, the low degradation rate of DNA, the easy quantification (as only one target per cell is present), and the stability throughout the disease course. However, the fusion genes frequently found in AML and CML have DNA breakpoints which are often scattered over large regions up to 200 kb and which are different in each patient. This implies that the identification of these breakpoint fusion sites is labor intensive, requiring special techniques (e.g. long-distance PCR, long-distance inverse PCR or ligation-mediated PCR). For small breakpoint regions (e.g. the *μ-bcr*), DNA targets can relatively easy be identified and potentially be used as patient-specific targets for monitoring of MRD.[290]

MRD detection by PCR analysis of Ig and TCR gene rearrangements

The junctional regions of rearranged Ig and TCR genes can be considered as "fingerprint-like" sequences, which are most probably different in each lymphocyte and thus also in each lymphoid malignancy.[291] Therefore, junctional regions can be used as leukemia-specific targets for MRD-PCR analysis. After confirming the clonality of the rearrangement and sequencing, junctional region-specific oligonucleotides can be designed and subsequently be tested for sensitivity and specificity using RQ-PCR analysis.[292] For this purpose, germline primers and probes have been designed during the last years for *IGH*, *IGK*-Kde, *TCRG*, *TCRD*, *TCRB*, and Vδ2-Jα rearrangements. These primer-probe sets in combination with junctional region-specific primers can generally reach sensitivities of 10^{-4}.[292] However, the sensitivity is dependent on the involved gene segments, size and sequence of the junctional region, and the background of comparable rearrangements in normal cells.[292]

Ig/TCR gene rearrangements might be prone to subclone formation, as shown in precursor-B-ALL. The problem of oligoclonality at diagnosis is the uncertainty which clone is going to emerge at relapse and should be monitored with MRD-PCR techniques. Moreover, secondary and ongoing Ig/TCR gene rearrangements might occur in the time period between diagnosis and relapse, resulting in the loss of leukemia-specific MRD targets. The design of primers around the relatively stable D-J region may prevent false-negative PCR results. In precursor-B-ALL, monoclonal MRD-PCR targets are characterized by a high stability (85-90% of all targets retained at relapse), whereas oligoclonal MRD-PCR targets are frequently lost (only 40% preserved at relapse).[293] So far, no data are available on the possible clonal evolution of Ig/TCR gene rearrangements in AML during therapy or at the time of relapse.

MRD detection by PCR analysis of other genetic aberrations

MRD detection via leukemia-specific fusion genes is only available in a minority of patients. However, the use of other genetic aberrations (e.g. *FLT3*-ITD or the overexpression of *WT1*) as molecular markers for MRD monitoring may potentially cover a large fraction of patients.

The detection of *FLT3*-ITD can be done both at the DNA and the RNA level.

Advantages of using *FLT3*-ITD as MRD-PCR targets are its patient specificity and the (theoretical) absence of background amplification in normal cells (in practice, some amplification in normal cells may be seen due to the limited specificity of the assay). Several studies reported on the instability of this marker during the course of the disease; in ~12% of patients gains or losses of *FLT3*-ITD at relapse are reported.[294-299] Consequently, *FLT3*-ITD should be regarded with caution with respect to its usefulness as MRD-PCR target.

WT1 may represent a molecular marker for MRD monitoring in the majority of patients, including the fusion gene transcript-negative AML patients. Aberrant *WT1* expression levels can easily be identified.[300-302] However, *WT1* expression is not patient-specific and expression of *WT1* in normal cells, which is dependent on the sample type (BM versus PB), may hamper a sensitive detection.[303-305]

3.2. CLINICAL RELEVANCE OF MRD DETECTION

For several categories of hematological malignancies, it has been shown that detection of MRD correlates with clinical outcome and that MRD information is important for clinical decision-making (Table 6).

Acute myeloid leukemia

A few large-scale studies evaluating the clinical relevance of MRD monitoring in AML have been performed. Studies in childhood AML, using flow cytometric immunophenotyping with cut-off levels between 0.1 and 0.5%, showed that patients with occult leukemia after induction therapy were significantly more likely to relapse

Table 6. Clinical applicability of MRD detection in myeloid malignancies.

	Type of MRD application			
	Early response to front-line treatment	*Continuous monitoring for therapy titration*	*MRD assessment before SCT*	*MRD assessment after SCT*
APL	++	++	+	+
AML	++	-	+	+
CML	+	+*	+	++

++ : value of MRD detection proven by prospective studies
+ : potentially clinically relevant but not yet proven by large prospective studies
- : MRD detection has no additional value as compared with conventional cytomorphological techniques
SCT : stem cell transplantation
* monitoring of treatment protocols which include imatinib

than those lacking detectable MRD.[276,306,307] Flow cytometric MRD studies in adult AML also have shown the clinical relevance of MRD, although the time point (after induction or at the end of consolidation) at which prognostic information was obtained differed between the reported studies.[275,277,308-311] Of importance are the studies by San Miguel *et al*., who defined different risk groups for AML patients in morphological remission according to the MRD level in BM after induction therapy. For an appropriate recognition of the true low-risk patients a sensitivity of 10^{-4} was required.[275,277] Such sensitivities cannot be obtained using a standard antibody panel but need patient-tailored labelings, depending on the LAIP's identified at diagnosis. However, in about 10-25% of patients no aberrant immunophenotype can be identified using the currently available technologies.[44,275]

MRD analysis in childhood and adult AML by PCR analysis of fusion gene transcripts has only been reported in relatively small patient series and generally showed that monitoring of MRD may be helpful in the recognition of patients at high risk of relapse and patients in a curable state.[312-323]

Acute promyelocytic leukemia

Particularly in APL, the clinical utility of frequent PCR-monitoring was confirmed in large prospective studies. After completing induction therapy including ATRA and chemotherapy, detectable *PML-RARA* transcripts were found in approximately 50% of patients.[225,324,325] No correlation was found between the PCR status at the time of morphological remission and the risk of relapse. By contrast, detection of MRD after the end of consolidation was strongly associated with subsequent overt hematological relapse, whereas the achievement of a PCR-negative status was associated with prolonged survival.[326] Early detection of molecular relapse, which usually precedes hematological relapse at a median time of 2-3 months, has been defined as a condition requiring early administration of salvage therapy; patients treated at the time point of molecular relapse had a significantly better 2-year event-free survival than patients treated with the same salvage therapy at the time of hematological relapse.[327,328] A number of small studies have highlighted the role of molecular monitoring in the context of hematopoietic stem cell transplantation (SCT) in APL. The pre-transplant status was a relevant indicator of subsequent outcome for patients undergoing *autologous* SCT,[329] while in patients undergoing an *allogeneic* SCT, PCR-positivity pre-transplant may convert to PCR-negativity after transplantation. Evidence to date suggest that patients with *PML-RARA* transcripts still detectable 3 months after SCT are destined to relapse in the absence of intervention,[329,330] while PCR-negativity has been consistently documented in BM derived from patients in long-term remission following autologous and allogeneic SCT.[330]

Chronic myeloid leukemia

For CML patients, much experience has been gained in measuring the *BCR-ABL* transcript levels in PB after conventional allogeneic SCT. The significance of

identifying *BCR-ABL* transcripts within the first 6 months after transplantation remains controversial. In one series such transcript numbers had no clinical relevance,[331] whereas in the Hammersmith experience, the absence of or presence of only low levels of residual disease early after SCT (at 3 to 5 months) was significantly correlated with a low risk of relapse at 3 years.[332] The finding of persisting *BCR-ABL* negativity from 6 months to 5 years after SCT predicted a low risk for subsequent relapse.[333] The *BCR-ABL* transcript levels were also informative after reduced-intensity SCT;[334] early increase of *BCR-ABL* transcripts correlated significantly with the probability of hematological relapse. In spite of the yet relatively short-term experiences, MRD information also seems of clinical importance in imatinib-treated CML patients. The IRIS trial investigated imatinib as primary therapy in CP CML patients: results showed that patients with a CCyR and a reduction in *BCR-ABL* transcript numbers of at least 3 log by 12 months of therapy, had a significantly superior progression-free survival as compared to those who achieved lesser degrees of molecular response or those who failed to achieve CCyR.[240,241] Longer follow-up should determine whether imatinib can induce prolonged progression-free survival and improved overall survival.

REFERENCES

1. Terstappen LW, Loken MR. Myeloid cell differentiation in normal bone marrow and acute myeloid leukemia assessed by multi-dimensional flow cytometry. *Anal Cell Patho* 1990; **2**: 229-240.
2. Terstappen LW, Safford M, Loken MR. Flow cytometric analysis of human bone marrow. III. Neutrophil maturation. *Leukemia* 1990; **4**: 657-663.
3. Loken MR, Shah VO, Dattilio KL, Civin CI. Flow cytometric analysis of human bone marrow: I. Normal erythroid development. *Blood* 1987; **69**: 255-263.
4. Loken MR, Wells DA. Normal Antigen Expression in hematopoiesis: basis for interpreting leukaemia phenotypes. Immunophenotyping 2000 Chapter 6.
5. Terstappen LW, Konemann S, Safford M, Loken MR, Zurlutter K, Buchner T, Hiddemann W, Wormann B. Flow cytometric characterization of acute myeloid leukemia. Part 1. Significance of light scattering properties. *Leukemia* 1991; **5**: 315-321.
6. Terstappen LW, Safford M, Konemann S, Loken MR, Zurlutter K, Buchner T, Hiddemann W, Wormann B. Flow cytometric characterization of acute myeloid leukemia. Part II. Phenotypic heterogeneity at diagnosis. *Leukemia* 1992; **6**: 70-80.
7. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposal for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol* 1976; **33**: 451-458.
8. San Miguel JF, Gonzalez M, Canizo MC, Anta JP, Zola H, Lopez Borrasca A. Surface marker analysis in acute myeloid leukaemia and correlation with FAB classification. *Br J Haematol* 1986; **64**: 547-560.
9. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, Van 't Veer MB. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia* 1995; **9**: 1783-1786.
10. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *J Clin Oncol* 1999; **17**: 3835-3849.

11. Villamor N, Zarco MA, Rozman M, Ribera JM, Feliu E, Montserrat E. Acute myeloblastic leukemia with minimal myeloid differentiation: phenotypical and ultrastructural characteristics. *Leukemia* 1998; **12**: 1071-1075.
12. Thalhammer-Scherrer R, Mitterbauer G, Simonitsch I, Jaeger U, Lechner K, Schneider B, Fonatsch C, Schwarzinger I. The immunophenotype of 325 adult acute leukemias: relationship to morphologic and molecular classification and proposal for a minimal screening program highly predictive for lineage discrimination. *Am J Clin Pathol* 2002; **117**: 380-389.
13. Kaleem Z, Crawford E, Pathan MH, Jasper L, Covinsky MA, Johnson LR, White G. Flow cytometric analysis of acute leukemias. Diagnostic utility and critical analysis of data. *Arch Pathol Lab Med* 2003; **127**: 42-48.
14. Venditti A, Del Poeta G, Buccisano F, Tamburini A, Cox MC, Stasi R, Bruno A, Aronica G, Maffei L, Suppo G, Simone MD, Forte L, Cordero V, Postorino M, Tufilli V, Isacchi G, Masi M, Papa G, Amadori S. Minimally differentiated acute myeloid leukemia (AML-M0): comparison of 25 cases with other French-American-British subtypes. *Blood* 1997; **89**: 621-629.
15. Scott CS, Richards SJ, Master PS, Kendall J, Limbert HJ, Roberts BE. Flow cytometric analysis of membrane CD11b, CD11c and CD14 expression in acute myeloid leukaemia: relationships with monocytic subtypes and the concept of relative antigen expression. *Eur J Haematol* 1990; **44**: 24-29.
16. Gassmann W, Loffler H. Acute megakaryoblastic leukemia. *Leuk Lymphoma* 1995; **18** Suppl 1: 69-73.
17. De Rossi G, Avvisati G, Coluzzi S, Fenu S, LoCoco F, Lopez M, Nanni M, Pasqualetti D, Mandelli F. Immunological definition of acute promyelocytic leukemia (FAB M3): a study of 39 cases. *Eur J Haematol* 1990; **45**: 168-171.
18. Vercauteren SM, Sutherland HJ. CD133 (AC133) expression on AML cells and progenitors. *Cytotherapy* 2001; **3**: 449-459.
19. Wuchter C, Ratei R, Spahn G, Schoch C, Harbott J, Schnittger S, Haferlach T, Creutzig U, Sperling C, Karawajew L, Ludwig WD. Impact of CD133 (AC133) and CD90 expression analysis for acute leukemia immunophenotyping. *Haematologica* 2001; **86**: 154-161.
20. Macedo A, Orfao A, Vidriales MB, Lopez-Berges MC, Valverde B, Gonzalez M, Caballero MD, Ramos F, Martinez M, Fernandez-Calvo J, *et al*. Characterization of aberrant phenotypes in acute myeloblastic leukemia. *Ann Hematol* 1995; **70**: 189-194.
21. Kern W, Danhauser-Riedl S, Ratei R, Schnittger S, Schoch C, Kolb HJ, Ludwig WD, Hiddemann W, Haferlach T. Detection of minimal residual disease in unselected patients with acute myeloid leukemia using multiparameter flow cytometry for definition of leukemia-associated immunophenotypes and determination of their frequencies in normal bone marrow. *Haematologica* 2003; **88**: 646-653.
22. Smith FO, Rauch C, Williams DE, March CJ, Arthur D, Hilden J, Lampkin BC, Buckley JD, Buckley CV, Woods WG, Dinndorf PA, Sorensen P, Kersey J, Hammond D, Bernstein ID. The human homologue of rat NG2, a chondroitin sulfate proteoglycan, is not expressed on the cell surface of normal hematopoietic cells but is expressed by acute myeloid leukemia blasts from poor-prognosis patients with abnormalities of chromosome band 11q23. *Blood* 1996; **87**: 1123-1133.
23. Hilden JM, Smith FO, Frestedt JL, McGlennen R, Howells WB, Sorensen PH, Arthur DC, Woods WG, Buckley J, Bernstein ID, Kersey JH. MLL gene rearrangement, cytogenetic 11q23 abnormalities, and expression of the NG2 molecule in infant acute myeloid leukemia. *Blood* 1997; **89**: 3801-3805.
24. Wuchter C, Harbott J, Schoch C, Schnittger S, Borkhardt A, Karawajew L, Ratei R, Ruppert V, Haferlach T, Creutzig U, Dorken B, Ludwig WD. Detection of acute leukemia cells with mixed lineage leukemia (*MLL*) gene rearrangements by flow cytometry using monoclonal antibody 7.1. *Leukemia* 2000; **14**: 1232-1238.

25. Khalidi HS, Medeiros LJ, Chang KL, Brynes RK, Slovak ML, Arber DA. The immunophenotype of adult acute myeloid leukemia: high frequency of lymphoid antigen expression and comparison of immunophenotype, French-American-British classification, and karyotypic abnormalities. *Am J Clin Pathol* 1998; **109**: 211-220.
26. Drexler HG, Sperling C, Ludwig WD. Terminal deoxynucleotidyl transferase (TdT) expression in acute myeloid leukemia. *Leukemia* 1993; **7**: 1142-1150.
27. Drexler HG, Thiel E, Ludwig WD. Acute myeloid leukemias expressing lymphoid-associated antigens: diagnostic incidence and prognostic significance. *Leukemia* 1993; **7**: 489-498.
28. Voskova D, Valekova L, Fedorova J, Hudecek J, Kubisz P. Leukemic cells and aberrant phenotypes in acute leukemia patients: a flow cytometry analysis. *Neoplasma* 2003; **50**: 422-427.
29. Macedo A, Orfao A, Gonzalez M, Vidriales MB, Lopez-Berges MC, Martinez A, San Miguel JF. Immunological detection of blast cell subpopulations in acute myeloblastic leukemia at diagnosis: implications for minimal residual disease studies. *Leukemia* 1995; **9**: 993-998.
30. Bene MC, Bernier M, Casasnovas RO, Castoldi G, Doekharan D, Van der Holt B, Knapp W, Lemez P, Ludwig WD, Matutes E, Orfao A, Schoch C, Sperling C, Van't Veer MB. Acute myeloid leukaemia M0: haematological, immunophenotypic and cytogenetic characteristics and their prognostic significance: an analysis in 241 patients. *Br J Haematol* 2001; **113**: 737-745.
31. Hrusak O, Porwit-MacDonald A. Antigen expression patterns reflecting genotype of acute leukemias. *Leukemia* 2002; **16**: 1233-1258.
32. Ferrara F, Di Noto R, Annunziata M, Copia C, Lo Pardo C, Boccuni P, Sebastio L, Del Vecchio L. Immunophenotypic analysis enables the correct prediction of t(8;21) in acute myeloid leukaemia. *Br J Haematol* 1998; **102**: 444-448.
33. Khoury H, Dalal BI, Nevill TJ, Horsman DE, Barnett MJ, Shepherd JD, Toze CL, Conneally EA, Sutherland HJ, Hogge DE, Nantel SH. Acute myelogenous leukemia with t(8;21)-identification of a specific immunophenotype. *Leuk Lymphoma* 2003; **44**: 1713-1718.
34. Orfao A, Chillon MC, Bortoluci AM, Lopez-Berges MC, Garcia-Sanz R, Gonzalez M, Tabernero MD, Garcia-Marcos MA, Rasillo AI, Hernandez-Rivas J, San Miguel JF. The flow cytometric pattern of CD34, CD15 and CD13 expression in acute myeloblastic leukemia is highly characteristic of the presence of *PML-RAR* alpha gene rearrangements. *Haematologica* 1999; **84**: 405-412.
35. Adriaansen HJ, te Boekhorst PA, Hagemeijer AM, Van der Schoot CE, Delwel HR, Van Dongen JJM. Acute myeloid leukemia M4 with bone marrow eosinophilia (M4Eo) and inv(16)(p13q22) exhibits a specific immunophenotype with CD2 expression. *Blood* 1993; **81**: 3043-3051.
36. Exner M, Thalhammer R, Kapiotis S, Mitterbauer G, Knobl P, Haas OA, Jager U, Schwarzinger I. The "typical" immunophenotype of acute promyelocytic leukemia (APL-M3): does it prove true for the M3-variant? *Cytometry* 2000; **42**: 106-109.
37. Mulford DA, Jurcic JG. Antibody-based treatment of acute myeloid leukaemia. *Expert Opin Biol Ther* 2004; **4**: 95-105.
38. Hamann PR, Hinman LM, Hollander I, Beyer CF, Lindh D, Holcomb R, Hallett W, Tsou HR, Upeslacis J, Shochat D, Mountain A, Flowers DA, Bernstein I. Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug Chem* 2002; **13**: 47-58.
39. Sievers EL. Antibody-targeted chemotherapy of acute myeloid leukemia using gemtuzumab ozogamicin (Mylotarg). *Blood Cells Mol Dis* 2003; **31**: 7-10.
40. Gadhoum Z, Delaunay J, Maquarre E, Durand L, Lancereaux V, Qi J, Robert-Lezenes J, Chomienne C, Smadja-Joffe F. The effect of anti-CD44 monoclonal antibodies on differentiation and proliferation of human acute myeloid leukemia cells. *Leuk Lymphoma* 2004; **45**: 1501-1510.
41. Gadhoum Z, Leibovitch MP, Qi J, Dumenil D, Durand L, Leibovitch S, Smadja-Joffe F. CD44: a new means to inhibit acute myeloid leukemia cell proliferation via p27Kip1. *Blood* 2004; **103**: 1059-1068.

42. Bakker AB, Van den Oudenrijn S, Bakker AQ, Feller N, Van Meijer M, Bia JA, Jongeneelen MA, Visser TJ, Bijl N, Geuijen CA, Marissen WE, Radosevic K, Throsby M, Schuurhuis GJ, Ossenkoppele GJ, De Kruif J, Goudsmit J, Kruisbeek AM. C-type lectin-like molecule-1: a novel myeloid cell surface marker associated with acute myeloid leukemia. *Cancer Res* 2004; **64**: 8443-8450.
43. Campana D, Coustan-Smith E. Detection of minimal residual disease in acute leukemia by flow cytometry. *Cytometry* 1999; **38**: 139-152.
44. San Miguel JF, Ciudad J, Vidriales MB, Orfao A, Lucio P, Porwit-MacDonald A, Gaipa G, Van Wering E, Van Dongen JJM. Immunophenotypical detection of minimal residual disease in acute leukemia. *Crit Rev Oncol Hematol* 1999; **32**: 175-185.
45. Szczepanski T, Orfao A, Van der Velden VHJ, San Miguel JF, Van Dongen JJM. Minimal residual disease in leukaemia patients. *Lancet Oncol* 2001; **2**: 409-417.
46. San-Miguel JF, Vidriales MB, Orfao A. Immunological evaluation of minimal residual disease (MRD) in acute myeloid leukaemia (AML). *Best Pract Res Clin Haematol* 2002; **15**: 105-18.
47. Nair C, Chopra H, Shinde S, Barbhaya S, Kumar A, Dhond S, Yejamanam B, Sapre R, Chougule A, Advani S. Immunophenotype and ultrastructural studies in blast crisis of chronic myeloid leukemia. *Leuk Lymphoma* 1995; **19**: 309-313.
48. Saikia T, Advani S, Dasgupta A, Ramakrishnan G, Nair C, Gladstone B, Kumar MS, Badrinath Y, Dhond S. Characterisation of blast cells during blastic phase of chronic myeloid leukaemia by immunophenotyping-experience in 60 patients. *Leuk Res* 1988; **12**: 499-506.
49. Khalidi HS, Brynes RK, Medeiros LJ, Chang KL, Slovak ML, Snyder DS, Arber DA. The immunophenotype of blast transformation of chronic myelogenous leukemia: a high frequency of mixed lineage phenotype in ”lymphoid” blasts and a comparison of morphologic, immunophenotypic, and molecular findings. *Mod Pathol* 1998; **11**: 1211-1221.
50. Okuda T, Takeda K, Fujita Y, Nishimura M, Yagyu S, Yoshida M, Akira S, Downing JR, Abe T. Biological characteristics of the leukemia-associated transcriptional factor AML1 disclosed by hematopoietic rescue of AML1-deficient embryonic stem cells by using a knock-in strategy. *Mol Cell Biol* 2000; **20**: 319-328.
51. Rowe D, Cotterill SJ, Ross FM, Bunyan DJ, Vickers SJ, Bryon J, McMullan DJ, Griffiths MJ, Reilly JT, Vandenberghe EA, Wilson G, Watmore AE, Bown NP. Cytogenetically cryptic *AML1-ETO* and *CBF beta-MYH11* gene rearrangements: incidence in 412 cases of acute myeloid leukaemia. *Br J Haematol* 2000; **111**: 1051-1056.
52. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, Rees J, Hann I, Stevens R, Burnett A, Goldstone A. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children’s Leukaemia Working Parties. *Blood* 1998; **92**: 2322-2333.
53. Van der Velden VHJ, Schoch C, Langerak AW, Schnittger S, Hoogeveen PG, Van Dongen JJM. Low frequency of reverse transcription polymerase chain reaction-detectable chromosome aberrations in relapsed acute myeloid leukaemia: implications for detection of minimal residual disease. *Br J Haematol* 2001; **113**: 1082-1083.
54. Van Dongen JJM, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G, Gottardi E, Rambaldi A, Dotti G, Griesinger F, Parreira A, Gameiro P, Diaz MG, Malec M, Langerak AW, San Miguel JF, Biondi A. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. *Leukemia* 1999; **13**: 1901-1928.
55. Liu PP, Hajra A, Wijmenga C, Collins FS. Molecular pathogenesis of the chromosome 16 inversion in the M4Eo subtype of acute myeloid leukemia. *Blood* 1995; **85**: 2289-2302.
56. Chillon CM, Garcia-Sanz R, Balanzategui A, Ramos F, Fernandez-Calvo J, Rodriguez MJ, Rodriguez-Salazar MI, Corrales A, Calmuntia MJ, Orfao A, Gonzalez M, San Miguel JF. Molecular characterization of acute myeloblastic leukemia according to the new WHO classification: a different distribution in Central-West Spain. *Haematologica* 2001; **86**: 162-166.

57. Pandolfi PP, Alcalay M, Fagioli M, Zangrilli D, Mencarelli A, Diverio D, Biondi A, Lo Coco F, Rambaldi A, Grignani F, *et al*. Genomic variability and alternative splicing generate multiple *PML/RAR* alpha transcripts that encode aberrant PML proteins and *PML/RAR* alpha isoforms in acute promyelocytic leukaemia. *EMBO J* 1992; **11**: 1397-1407.
58. Zelent A, Guidez F, Melnick A, Waxman S, Licht JD. Translocations of the *RAR*alpha gene in acute promyelocytic leukemia. *Oncogene* 2001; **20**: 7186-7203.
59. Pui CH, Kane JR, Crist WM. Biology and treatment of infant leukemias. *Leukemia* 1995; **9**: 762-769.
60. Pui CH, Raimondi SC, Srivastava DK, Tong X, Behm FG, Razzouk B, Rubnitz JE, Sandlund JT, Evans WE, Ribeiro R. Prognostic factors in infants with acute myeloid leukemia. *Leukemia* 2000; **14**: 684-687.
61. Macintyre E, Bourquelot P, Leboeuf D, Rimokh R, Archimbaud E, Smetsers T, Zittoun R. MLL cleavage occurs in approximately 5% of *de novo* acute myeloid leukemia, including in patients analyzed before treatment induction. *Blood* 1997; **89**: 2224-2226.
62. Super HJ, McCabe NR, Thirman MJ, Larson RA, Le Beau MM, Pedersen-Bjergaard J, Philip P, Diaz MO, Rowley JD. Rearrangements of the *MLL* gene in therapy-related acute myeloid leukemia in patients previously treated with agents targeting DNA-topoisomerase II. *Blood* 1993; **82**: 3705-3711.
63. Huret JL, Dessen P, Bernheim A. An atlas of chromosomes in hematological malignancies. Example: 11q23 and *MLL* partners. *Leukemia* 2001; **15**: 987-989.
64. Huret JL. *MLL* (myeloid/lymphoid or mixed lineage leukemia). Atlas Genet Cytogenet Oncol Haematol 2005. http://www.infobiogen.fr/services/chromcancer/Genes/MLL.html.
65. Meyer C, Schneider B, Jacob S, Strehl S, Attarbaschi A, Schnittger S, Schoch C, Jansen MWJ, Van Dongen JJM, Den Boer ML, Pieters R, Ennas M, Angelucci E, Koehl U, Greil J, Griesinger F, zur Stadt U, Eckert C, Szczepanski T, Niggli FK, Schäfer BW, Kempski H, Brady HJM, Zuna J, Trka J, Lo Nigro L, Biondi A, Delabesse E, Macintyre E, Stanulla M, Schrappe M, Haas OA, Burmeister T, Dingermann T, Klingebiel T, Marschalek R. The MLL recombinome of acute leukemia. Submitted.
66. Harrison CJ, Cuneo A, Clark R, Johansson B, Lafage-Pochitaloff M, Mugneret F, Moorman AV, Secker-Walker LM. Ten novel 11q23 chromosomal partner sites. European 11q23 Workshop participants. *Leukemia* 1998; **12**: 811-822.
67. Moorman AV, Hagemeijer A, Charrin C, Rieder H, Secker-Walker LM. The translocations, t(11;19)(q23;p13.1) and t(11;19)(q23;p13.3): a cytogenetic and clinical profile of 53 patients. European 11q23 Workshop participants. *Leukemia* 1998; **12**: 805-810.
68. Swansbury GJ, Slater R, Bain BJ, Moorman AV, Secker-Walker LM. Hematological malignancies with t(9;11)(p21-22;q23)-a laboratory and clinical study of 125 cases. European 11q23 Workshop participants. *Leukemia* 1998; **12**: 792-800.
69. Lillington DM, Young BD, Berger R, Martineau M, Moorman AV, Secker-Walker LM. The t(10;11)(p12;q23) translocation in acute leukaemia: a cytogenetic and clinical study of 20 patients. European 11q23 Workshop participants. *Leukemia* 1998; **12**: 801-804.
70. Martineau M, Berger R, Lillington DM, Moorman AV, Secker-Walker LM. The t(6;11)(q27;q23) translocation in acute leukemia: a laboratory and clinical study of 30 cases. EU Concerted Action 11q23 Workshop participants. *Leukemia* 1998; **12**: 788-791.
71. Rubnitz JE, Raimondi SC, Tong X, Srivastava DK, Razzouk BI, Shurtleff SA, Downing JR, Pui CH, Ribeiro RC, Behm FG. Favorable impact of the t(9;11) in childhood acute myeloid leukemia. *J Clin Oncol* 2002; **20**: 2302-2309.
72. Martinez-Climent JA, Lane NJ, Rubin CM, Morgan E, Johnstone HS, Mick R, Murphy SB, Vardiman JW, Larson RA, Le Beau MM, *et al*. Clinical and prognostic significance of chromosomal abnormalities in childhood acute myeloid leukemia *de novo*. *Leukemia* 1995; **9**: 95-101.

73. Mrozek K, Heinonen K, Lawrence D, Carroll AJ, Koduru PR, Rao KW, Strout MP, Hutchison RE, Moore JO, Mayer RJ, Schiffer CA, Bloomfield CD. Adult patients with *de novo* acute myeloid leukemia and t(9;11)(p22;q23) have a superior outcome to patients with other translocations involving band 11q23: a cancer and leukemia group B study. *Blood* 1997; **90**: 4532-4538.
74. Raimondi SC, Chang MN, Ravindranath Y, Behm FG, Gresik MV, Steuber CP, Weinstein HJ, Carroll AJ. Chromosomal abnormalities in 478 children with acute myeloid leukemia: clinical characteristics and treatment outcome in a cooperative pediatric oncology group study-POG 8821. *Blood* 1999; **94**: 3707-3716.
75. Wells RJ, Arthur DC, Srivastava A, Heerema NA, Le Beau M, Alonzo TA, Buxton AB, Woods WG, Howells WB, Benjamin DR, Betcher DL, Buckley JD, Feig SA, Kim T, Odom LF, Ruymann FB, Smithson WA, Tannous R, Whitt JK, Wolff L, Tjoa T, Lampkin BC. Prognostic variables in newly diagnosed children and adolescents with acute myeloid leukemia: Children's Cancer Group Study 213. *Leukemia* 2002; **16**: 601-607.
76. Hanslip JI, Swansbury GJ, Pinkerton R, Catovsky D. The translocation t(8;16)(p11;p13) defines an AML subtype with distinct cytology and clinical features. *Leuk Lymphoma* 1992; **6**: 479-486.
77. Velloso ER, Mecucci C, Michaux L, Van Orshoven A, Stul M, Boogaerts M, Bosly A, Cassiman JJ, Van Den Berghe H. Translocation t(8;16)(p11;p13) in acute non-lymphocytic leukemia: report on two new cases and review of the literature. *Leuk Lymphoma* 1996; **21**: 137-142.
78. Soekarman D, Von Lindern M, Daenen S, De Jong B, Fonatsch C, Heinze B, Bartram C, Hagemeijer A, Grosveld G. The translocation (6;9) (p23;q34) shows consistent rearrangement of two genes and defines a myeloproliferative disorder with specific clinical features. *Blood* 1992; **79**: 2990-2997.
79. Rubin CM, Larson RA, Anastasi J, Winter JN, Thangavelu M, Vardiman JW, Rowley JD, Le Beau MM. t(3;21)(q26;q22): a recurring chromosomal abnormality in therapy-related myelodysplastic syndrome and acute myeloid leukemia. *Blood* 1990; **76**: 2594-2598.
80. Kong XT, Ida K, Ichikawa H, Shimizu K, Ohki M, Maseki N, Kaneko Y, Sako M, Kobayashi Y, Tojou A, Miura I, Kakuda H, Funabiki T, Horibe K, Hamaguchi H, Akiyama Y, Bessho F, Yanagisawa M, Hayashi Y. Consistent detection of *TLS/FUS-ERG* chimeric transcripts in acute myeloid leukemia with t(16;21)(p11;q22) and identification of a novel transcript. *Blood* 1997; **90**: 1192-1199.
81. Gilliland DG, Griffin JD. The roles of *FLT3* in hematopoiesis and leukemia. *Blood* 2002; **100**: 1532-1542.
82. Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, Loffler H, Sauerland CM, Serve H, Buchner T, Haferlach T, Hiddemann W. Analysis of *FLT3* length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood* 2002; **100**: 59-66.
83. Moreno I, Martin G, Bolufer P, Barragan E, Rueda E, Roman J, Fernandez P, Leon P, Mena A, Cervera J, Torres A, Sanz MA. Incidence and prognostic value of *FLT3* internal tandem duplication and D835 mutations in acute myeloid leukemia. *Haematologica* 2003; **88**: 19-24.
84. Kiyoi H, Towatari M, Yokota S, Hamaguchi M, Ohno R, Saito H, Naoe T. Internal tandem duplication of the *FLT3* gene is a novel modality of elongation mutation which causes constitutive activation of the product. *Leukemia* 1998; **12**: 1333-1337.
85. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Saito K, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Saito H, Ueda R, Ohno R, Naoe T. Activating mutation of D835 within the activation loop of *FLT3* in human hematologic malignancies. *Blood* 2001; **97**: 2434-2439.
86. Abu-Duhier FM, Goodeve AC, Wilson GA, Care RS, Peake IR, Reilly JT. Identification of novel *FLT-3* Asp835 mutations in adult acute myeloid leukaemia. *Br J Haematol* 2001; **113**: 983-988.

87. Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U, Wermke M, Bornhauser M, Ritter M, Neubauer A, Ehninger G, Illmer T. Analysis of *FLT3*-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* 2002; **99**: 4326-4335.
88. Spiekermann K, Bagrintseva K, Schoch C, Haferlach T, Hiddemann W, Schnittger S. A new and recurrent activating length mutation in exon 20 of the *FLT3* gene in acute myeloid leukemia. *Blood* 2002; **100**: 3423-3425.
89. Jiang J, Paez JG, Lee JC, Bo R, Stone RM, DeAngelo DJ, Galinsky I, Wolpin BM, Jonasova A, Herman P, Fox EA, Boggon TJ, Eck MJ, Weisberg E, Griffin JD, Gilliland DG, Meyerson M, Sellers WR. Identifying and characterizing a novel activating mutation of the *FLT3* tyrosine kinase in AML. *Blood* 2004; **104**: 1855-1858.
90. Kindler T, Breitenbuecher F, Kasper S, Estey E, Giles F, Feldman E, Ehninger G, Schiller G, Klimek V, Nimer SD, Gratwohl A, Choudhary CR, Mueller-Tidow C, Serve H, Gschaidmeier H, Cohen PS, Huber C, Fischer T. Identification of a novel activating mutation (Y842C) within the activation loop of *FLT3* in patients with acute myeloid leukemia (AML). *Blood* 2005; **105**: 335-340.
91. Meshinchi S, Woods WG, Stirewalt DL, Sweetser DA, Buckley JD, Tjoa TK, Bernstein ID, Radich JP. Prevalence and prognostic significance of *FLT3* internal tandem duplication in pediatric acute myeloid leukemia. *Blood* 2001; **97**: 89-94.
92. Rombouts WJ, Blokland I, Löwenberg B, Ploemacher RE. Biological characteristics and prognosis of adult acute myeloid leukemia with internal tandem duplications in the *FLT3* gene. *Leukemia* 2000; **14**: 675-683.
93. Shih LY, Kuo MC, Liang DC, Huang CF, Lin TL, Wu JH, Wang PN, Dunn P, Lai CL. Internal tandem duplication and Asp835 mutations of the FMS-like tyrosine kinase 3 (*FLT3*) gene in acute promyelocytic leukemia. *Cancer* 2003; **98**: 1206-1216.
94. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, Asou N, Kuriyama K, Jinnai I, Shimazaki C, Akiyama H, Saito K, Oh H, Motoji T, Omoto E, Saito H, Ohno R, Ueda R. Prognostic implication of *FLT3* and *N-RAS* gene mutations in acute myeloid leukemia. *Blood* 1999; **93**: 3074-3080.
95. Kondo M, Horibe K, Takahashi Y, Matsumoto K, Fukuda M, Inaba J, Kato K, Kojima S, Matsuyama T. Prognostic value of internal tandem duplication of the *FLT3* gene in childhood acute myelogenous leukemia. *Med Pediatr Oncol* 1999; **33**: 525-529.
96. Meshinchi S, Woods WG, Stirewalt DL, Sweetser DA, Buckley JD, Tjoa TK, Bernstein ID, Radich JP. Prevalence and prognostic significance of *FLT3* internal tandem duplication in pediatric acute myeloid leukemia. *Blood* 2001; **97**: 89-94.
97. Zwaan CM, Meshinchi S, Radich JP, Veerman AJ, Huismans DR, Munske L, Podleschny M, Hahlen K, Pieters R, Zimmermann M, Reinhardt D, Harbott J, Creutzig U, Kaspers GJ, Griesinger F. *FLT3* internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. *Blood* 2003; **102**: 2387-2394.
98. Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, Walker H, Wheatley K, Bowen DT, Burnett AK, Goldstone AH, Linch DC. The presence of a *FLT3* internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood* 2001; **98**: 1752-1759.
99. Ciolli S, Vannucchi AM, Leoni F, Nozzoli C, Longo G, Salati A, Pancrazzi A, Bianchi L, Gigli F, Bosi A. Internal tandem duplications of *FLT3* gene (*FLT3*/ITD) predicts a poor post-remission outcome in adult patients with acute non-promyelocytic leukemia. *Leuk Lymphoma* 2004; **45**: 73-78.
100. Au WY, Fung A, Chim CS, Lie AK, Liang R, Ma ES, Chan CH, Wong KF, Kwong YL. *FLT-3* aberrations in acute promyelocytic leukaemia: clinicopathological associations and prognostic impact. *Br J Haematol* 2004; **125**: 463-469.

101. Stirewalt DL, Kopecky KJ, Meshinchi S, Appelbaum FR, Slovak ML, Willman CL, Radich JP. *FLT3*, *RAS*, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood* 2001; **97**: 3589-3595.
102. Barletta E, Gorini G, Vineis P, Miligi L, Davico L, Mugnai G, Ciolli S, Leoni F, Bertini M, Matullo G, Costantini AS. *RAS* gene mutations in patients with acute myeloid leukaemia and exposure to chemical agents. *Carcinogenesis* 2004; **25**: 749-755.
103. Gougopoulou DM, Kiaris H, Ergazaki M, Anagnostopoulos NI, Grigoraki V, Spandidos DA. Mutations and expression of the *RAS* family genes in leukemias. *Stem Cells* 1996; **14**: 725-729.
104. Neubauer A, Dodge RK, George SL, Davey FR, Silver RT, Schiffer CA, Mayer RJ, Ball ED, Wurster-Hill D, Bloomfield CD, *et al*. Prognostic importance of mutations in the *RAS* proto-oncogenes in *de novo* acute myeloid leukemia. *Blood* 1994; **83**: 1603-1611.
105. Farr CJ, Saiki RK, Erlich HA, McCormick F, Marshall CJ. Analysis of *RAS* gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes. *Proc Natl Acad Sci USA* 1988; **85**: 1629-1633.
106. Coghlan DW, Morley AA, Matthews JP, Bishop JF. The incidence and prognostic significance of mutations in codon 13 of the *N-RAS* gene in acute myeloid leukemia. *Leukemia* 1994; **8**: 1682-1687.
107. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, Asou N, Kuriyama K, Jinnai I, Shimazaki C, Akiyama H, Saito K, Oh H, Motoji T, Omoto E, Saito H, Ohno R, Ueda R. Prognostic implication of *FLT3* and *N-RAS* gene mutations in acute myeloid leukemia. *Blood* 1999; **93**: 3074-3080.
108. Liang DC, Shih LY, Huang CF, Hung IJ, Yang CP, Liu HC, Jaing TH, Wang LY, Chang WH. *CEBP*alpha mutations in childhood acute myeloid leukemia. *Leukemia* 2005; **19**: 410-414.
109. Frohling S, Schlenk RF, Stolze I, Bihlmayr J, Benner A, Kreitmeier S, Tobis K, Dohner H, Dohner K. *CEBPA* mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. *J Clin Oncol* 2004; **22**: 624-633.
110. Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C. *CEBPA* point mutations in hematological malignancies. *Leukemia* 2005; **19**: 329-334.
111. Osata M. Point mutations in the *RUNX1/AML1* gene: another actor in RUNX leukemia. *Oncogene* 2004; **23**: 4284-4296.
112. Matsuno N, Osato M, Yamashita N, Yanagida M, Nanri T, Fukushima T, Motoji T, Kusumoto S, Towatari M, Suzuki R, Naoe T, Nishii K, Shigesada K, Ohno R, Mitsuya H, Ito Y, Asou N. Dual mutations in the *AML1* and *FLT3* genes are associated with leukemogenesis in acute myeloblastic leukemia of the M0 subtype. *Leukemia* 2003; **17**: 2492-2499.
113. Roumier C, Eclache V, Imbert M, Davi F, MacIntyre E, Garand R, Talmant P, Lepelley P, Lai JL, Casasnovas O, Maynadie M, Mugneret F, Bilhou-Naberra C, Valensi F, Radford I, Mozziconacci MJ, Arnoulet C, Duchayne E, Dastugue N, Cornillet P, Daliphard S, Garnache F, Boudjerra N, Jouault H, Fenneteau O, Pedron B, Berger R, Flandrin G, Fenaux P, Preudhomme C; Groupe Francais de Cytogenetique Hematologique (GFCH); Groupe Francais d'Hematologie Cellulaire (GFHC). M0 AML, clinical and biologic features of the disease, including *AML1* gene mutations: a report of 59 cases by the Groupe Français d'Haematologie Cellulaire (GFHC) and the Groupe Français de Cytogenetique Haematologique (GFCH). *Blood* 2003; **101**: 1277-1283.
114. Osato M, Asou N, Abdalla E, Hoshino K, Yamasaki H, Okubo T, Suzushima H, Takatsuki K, Kanno T, Shigesada K, Ito Y. Biallelic and heterozygous point mutations in the runt domain of the *AML1/PEBP2*alphaB gene associated with myeloblastic leukemias. *Blood* 1999; **93**: 1817-1824.
115. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, La Starza R, Diverio D, Colombo E, Santucci A, Bigerna B, Pacini R, Pucciarini A, Liso A, Vignetti M, Fazi P, Meani N, Pettirossi V, Saglio G, Mandelli F, Lo-Coco F, Pelicci PG, Martelli MF. GIMEMA Acute Leukemia Working Party. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med* 2005; **352**: 254-266.

116. Cazzaniga G, Dell'Oro MG, Mecucci G, Giarin E, Masetti R, Rossi V, Locatelli F, Martelli MF, Basso G, Pession A, Biondi A, Falini B. Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. *Blood* 2005; **106**: 1419-1422.
117. Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C, Martelli MF, Haferlach T, Hiddemann W, Falini B. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood* 2005; **106**: 3733-3739.
118. Dohner K, Schlenk RF, Habdank M, Scholl C, Rucker FG, Corbacioglu A, Bullinger L, Frohling S, Dohner H. Mutant nucleophosmin (*NPM1*) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood* 2005; **106**: 3740-3746.
119. Verhaak RG, Goudswaard CS, Van Putten W, Bijl MA, Sanders MA, Hugens W, Uitterlinden AG, Erpelinck CA, Delwel R, Löwenberg B, Valk PJ. Mutations in nucleophosmin (*NPM1*) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood* 2005; **106**: 3747-3754.
120. Boissel N, Renneville A, Biggio V, Philippe N, Thomas X, Cayuela JM, Terre C, Tigaud I, Castaigne S, Raffoux E, De Botton S, Fenaux P, Dombret H, Preudhomme C. Prevalence, clinical profile, and prognosis of *NPM* mutations in AML with normal karyotype. *Blood* 2005; **106**: 3618-3620.
121. Menssen HD, Renkl HJ, Rodeck U, Maurer J, Notter M, Schwartz S, Reinhardt R, Thiel E. Presence of Wilms' tumor gene (*wt1*) transcripts and the *WT1* nuclear protein in the majority of human acute leukemias. *Leukemia* 1995; **9**: 1060-1067.
122. Steinbach D, Hermann J, Viehmann S, Zintl F, Gruhn B. Clinical implications of *PRAME* gene expression in childhood acute myeloid leukemia. *Cancer Genet Cytogenet* 2002; **133**: 118-123.
123. Van Baren N, Chambost H, Ferrant A, Michaux L, Ikeda H, Millard I, Olive D, Boon T, Coulie PG. *PRAME*, a gene encoding an antigen recognized on a human melanoma by cytolytic T cells, is expressed in acute leukaemia cells. *Br J Haematol* 1998; **102**: 1376-1379.
124. Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R, Kantarjian HM. The biology of chronic myeloid leukemia. *N Engl J Med* 1999; **341**: 164-172.
125. De Klein A, Van Kessel AG, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, Spurr NK, Heisterkamp N, Groffen J, Stephenson JR. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* 1982; **300**: 765-767.
126. Salesse S, Verfaillie CM. *BCR/ABL*: from molecular mechanisms of leukemia induction to treatment of chronic myelogenous leukemia. *Oncogene* 2002; **21**: 8547-8559.
127. Kurzrock R, Gutterman JU, Talpaz M. The molecular genetics of Philadelphia chromosome-positive leukemias. *N Engl J Med* 1988; **319**: 990-998.
128. Groffen J, Stephenson JR, Heisterkamp N, De Klein A, Bartram CR, Grosveld G. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell* 1984; **36**: 93-99.
129. Pane F, Frigeri F, Sindona M, Luciano L, Ferrara F, Cimino R, Meloni G, Saglio G, Salvatore F, Rotoli B. Neutrophilic-chronic myeloid leukemia: a distinct disease with a specific molecular marker (*BCR/ABL* with c3/a2 junction). *Blood* 1996; **88**: 2410-2414.
130. Saglio G, Rosso C, Zaccaria A, Tassinari A, Serra A, Rege-Cambrin G, Mazza U, Gavosto F. New type of *Bcr/Abl* junction in Philadelphia chromosome-positive chronic myelogenous leukemia. *Blood* 1990; **76**: 1819-1824.
131. Melo JV. The diversity of *BCR-ABL* fusion proteins and their relationship to leukemia phenotype. *Blood* 1996; **88**: 2375-2384.
132. Melo JV, Myint H, Galton DA, Goldman JM. P190*BCR-ABL* chronic myeloid leukaemia: the missing link with chronic myelomonocytic leukaemia? *Leukemia* 1994; **8**: 208-211.

133. Selleri L, Von Lindern M, Hermans A, Meijer D, Torelli G, Grosveld G. Chronic myeloid leukemia may be associated with several *BCR-ABL* transcripts including the acute lymphoid leukemia-type 7 kb transcript. *Blood* 1990; **75**: 1146-1153.
134. Leibundgut EO, Jotterand M, Rigamonti V, Parlier V, Muhlematter D, Tobler A, Solenthaler M. A novel *BCR-ABL* transcript e2a2 in a chronic myelogenous leukaemia patient with a duplicated Ph-chromosome and monosomy 7. *Br J Haematol* 1999; **106**: 1041-1044.
135. Hochhaus A, Reiter A, Skladny H, Melo JV, Sick C, Berger U, Guo JQ, Arlinghaus RB, Hehlmann R, Goldman JM, Cross NC. A novel *BCR-ABL* fusion gene (e6a2) in a patient with Philadelphia chromosome-negative chronic myelogenous leukemia. *Blood* 1996; **88**: 2236-2240.
136. How GF, Lim LC, Kulkarni S, Tan LT, Tan P, Cross NC. Two patients with novel *BCR/ABL* fusion transcripts (e8/a2 and e13/a2) resulting from translocation breakpoints within *BCR* exons. *Br J Haematol* 1999; **105**: 434-436.
137. Al-Ali HK, Leiblein S, Kovacs I, Hennig E, Niederwieser D, Deininger MW. CML with an e1a3 *BCR-ABL* fusion: rare, benign, and a potential diagnostic pitfall. *Blood* 2002; **100**: 1092-1093.
138. Branford S, Rudzki Z, Hughes TP. A novel *BCR-ABL* transcript (e8a2) with the insertion of an inverted sequence of *ABL* intron 1b in a patient with Philadelphia-positive chronic myeloid leukaemia. *Br J Haematol* 2000; **109**: 635-637.
139. Shiratsuchi M, Muta K, Minami R, Motomura S, Suehiro Y, Abe Y, Shiokawa S, Umemura T, Fukui T, Nishimura J, Nawata H. Aberrant *BCR-ABL* transcript with intronic insertion in a patient with philadelphia chromosome-positive chronic myeloid leukemia: implications for disease progression. *Leuk Lymphoma* 2001; **41**: 411-415.
140. Biernaux C, Sels A, Huez G, Stryckmans P. Very low level of major *BCR-ABL* expression in blood of some healthy individuals. *Bone Marrow Transplant* 1996; **17**: S45-47.
141. Bose S, Deininger M, Gora-Tybor J, Goldman JM, Melo JV. The presence of typical and atypical *BCR-ABL* fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. *Blood* 1998; **92**: 3362-3367.
142. Van Rhee F, Hochhaus A, Lin F, Melo JV, Goldman JM, Cross NC. p190 *BCR-ABL* mRNA is expressed at low levels in p210-positive chronic myeloid and acute lymphoblastic leukemias. *Blood* 1996; **87**: 5213-5217.
143. Lichty BD, Keating A, Callum J, Yee K, Croxford R, Corpus G, Nwachukwu B, Kim P, Guo J, Kamel-Reid S. Expression of p210 and p190 *BCR-ABL* due to alternative splicing in chronic myelogenous leukaemia. *Br J Haematol* 1998; **103**: 711-715.
144. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, *RAG-1*. *Cell* 1989; **59**: 1035-1048.
145. Oettinger MA, Schatz DG, Gorka C, Baltimore D. *RAG-1* and *RAG-2*, adjacent genes that synergistically activate V(D)J recombination. *Science* 1990; **248**: 1517-1523.
146. Lieber MR. The mechanism of V(D)J recombination: a balance of diversity, specificity, and stability. *Cell* 1992; **70**: 873-876.
147. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW, Baltimore D. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. *Nature* 1984; **311**: 752-755.
148. Krejci O, Prouzova Z, Horvath O, Trka J, Hrusak O. Cutting edge: TCR delta gene is frequently rearranged in adult B lymphocytes. *J Immunol* 2003; **171**: 524-527.
149. Szczepanski T, Beishuizen A, Pongers-Willemse MJ, Hahlen K, Van Wering ER, Wijkhuijs AJ, Tibbe GJ, De Bruijn MA, Van Dongen JJM. Cross-lineage T cell receptor gene rearrangements occur in more than ninety percent of childhood precursor-B acute lymphoblastic leukemias: alternative PCR targets for detection of minimal residual disease. *Leukemia* 1999; **13**: 196-205.
150. Szczepanski T, Pongers-Willemse MJ, Langerak AW, Harts WA, Wijkhuijs AJ, Van Wering ER, Van Dongen JJM. Ig heavy chain gene rearrangements in T-cell acute lymphoblastic leukemia exhibit predominant DH6-19 and DH7-27 gene usage, can result in complete V-D-J rearrangements, and are rare in T-cell receptor alpha beta lineage. *Blood* 1999; **93**: 4079-4085.

151. Adriaansen HJ, Soeting P, Wolvers-Tettero IL, Van Dongen JJM. Immunoglobulin and T-cell receptor gene rearrangements in acute non-lymphocytic leukemias. Analysis of 54 cases and a review of the literature. *Leukemia* 1991; **5**: 744-751.
152. Boeckx N, Willemse MJ, Szczepanski T, Van der Velden VHJ, Langerak AW, Vandekerckhove P, Van Dongen JJM. Fusion gene transcripts and Ig/TCR gene rearrangements are complementary but infrequent targets for PCR-based detection of minimal residual disease in acute myeloid leukemia. *Leukemia* 2002; **16**: 368-375.
153. Sanchez I, San Miguel J, Corral J, Martin C, Perez R, Gonzalez M, Canizo MC, Orfao A, Gonzalez-Sarmiento R. Gene rearrangement in acute non-lymphoblastic leukaemia: correlation with morphological and immunophenotypic characteristics of blast cells. *Br J Haematol* 1995; **89**: 104-109.
154. Schmidt CA, Oettle H, Neubauer A, Seeger K, Thiel E, Huhn D, Siegert W, Ludwig WD. Rearrangements of T-cell receptor delta, gamma and beta genes in acute myeloid leukemia coexpressing T-lymphoid features. *Leukemia* 1992; **6**: 1263-1267.
155. Stavroyianni N, Belessi C, Stamatopoulos K, Kosmas C, Paterakis G, Abazis D, Pangalos C, Yataganas X; Greek AML Study Group. Expression of recombination activating genes-1 and -2 immunoglobulin heavy chain gene rearrangements in acute myeloid leukemia: evaluation of biological and clinical significance in a series of 76 uniformly treated patients and review of the literature. *Haematologica* 2003; **88**: 268-274.
156. Kyoda K, Nakamura S, Matano S, Ohtake S, Matsuda T. Prognostic significance of immunoglobulin heavy chain gene rearrangement in patients with acute myelogenous leukemia. *Leukemia* 1997; **11**: 803-806.
157. Yen CC, Liu JH, Wang WS, Chiou TJ, Fan FS, Chen PM. Prognostic significance of immunoglobulin and T cell receptor gene rearrangements in patients with acute myeloid leukemia: Taiwan experience. *Leuk Lymphoma* 1999; **35**: 179-187.
158. Foa R, Casorati G, Giubellino MC, Basso G, Schiro R, Pizzolo G, Lauria F, Lefranc MP, Rabbitts TH, Migone N. Rearrangements of immunoglobulin and T cell receptor beta and gamma genes are associated with terminal deoxynucleotidyl transferase expression in acute myeloid leukemia. *J Exp Med* 1987; **165**: 879-890.
159. Schmidt CA, Przybylski G, Seeger K, Siegert W. TCR delta gene rearrangements in acute myeloid leukemia with T-lymphoid antigen expression. *Leuk Lymphoma* 1995; **20**: 45-49.
160. Di Celle PF, Carbone A, Lo Coco F, Guerrasio A, Diverio D, Rosso C, Lopez M, Saglio G, Foa R. Identical utilization of T-cell receptor gene regions in B-lymphoid blast crisis of chronic myeloid leukemia and B-precursor acute lymphoblastic leukemia. *Leukemia* 1991; **5**: 366-372.
161. Kerim S, Stul M, Mecucci C, Vandenberghe E, Cuneo A, Cin PD, Michaux JL, Louwagie A, Cassiman JJ, Van den Berghe H. Rearrangement of immunoglobulin and TCR genes in lymphoid blast crisis of Ph+ chronic myeloid leukaemia. *Br J Haematol* 1990; **74**: 414-419.
162. Bashey A, Vulliamy T, Cai BS, Schwarer AP, Goldman JM. Non-lymphoid blast crisis of CML with rearrangement of immunoglobulin and T-cell receptor delta genes. *Eur J Haematol* 1991; **47**: 36-41.
163. Spencer A, Vulliamy T, Kaeda J, Goldman JM, Melo JV. Clonal instability preceding lymphoid blastic transformation of chronic myeloid leukemia. *Leukemia* 1997; **11**: 195-201.
164. Spencer A, Szydlo R, Grand FH, Goldman JM, Melo JV. Abnormal patterns of immunoglobulin heavy chain DNA fingerprinting during chronic phase chronic myeloid leukemia. *Leuk Lymphoma* 1999; **32**: 299-307.
165. Hann IM, Stevens RF, Goldstone AH, Rees JK, Wheatley K, Gray RG, Burnett AK. Randomized comparison of DAT versus ADE as induction chemotherapy in children and younger adults with acute myeloid leukemia. Results of the Medical Research Council's 10th AML trial (MRC AML10). Adult and Childhood Leukaemia Working Parties of the Medical Research Council. *Blood* 1997; **89**: 2311-2318.

166. Woods WG, Kobrinsky N, Buckley JD, Lee JW, Sanders J, Neudorf S, Gold S, Barnard DR, DeSwarte J, Dusenbery K, Kalousek D, Arthur DC, Lange BJ. Timed-sequential induction therapy improves postremission outcome in acute myeloid leukemia: a report from the Children's Cancer Group. *Blood* 1996; **87**: 4979-4989.
167. Creutzig U, Ritter J, Zimmermann M, Reinhardt D, Hermann J, Berthold F, Henze G, Jurgens H, Kabisch H, Havers W, Reiter A, Kluba U, Niggli F, Gadner H. Improved treatment results in high-risk pediatric acute myeloid leukemia patients after intensification with high-dose cytarabine and mitoxantrone: results of Study Acute Myeloid Leukemia-Berlin-Frankfurt-Munster 93. *J Clin Oncol* 2001; **19**: 2705-2713.
168. Goldstone AH, Burnett AK, Wheatley K, Smith AG, Hutchinson RM, Clark RE; Medical Research Council Adult Leukemia Working Party. Attempts to improve treatment outcomes in acute myeloid leukemia (AML) in older patients: the results of the United Kingdom Medical Research Council AML11 trial. *Blood* 2001; **98**: 1302-1311.
169. Löwenberg B, Downing JR, Burnett A. Acute myeloid leukemia. *N Engl J Med* 1999; **341**: 1051-1062.
170. Gorin NC. Autologous stem cell transplantation in acute myelocytic leukemia. *Blood* 1998; **92**: 1073-1090.
171. Michel G, Leverger G, Leblanc T, Nelken B, Baruchel A, Landman-Parker J, Thuret I, Bergeron C, Bordigoni P, Esperou-Bourdeau H, Perel Y, Vannier JP, Schaison G. Allogeneic bone marrow transplantation vs aggressive post-remission chemotherapy for children with acute myeloid leukemia in first complete remission. A prospective study from the French Society of Pediatric Hematology and Immunology (SHIP). *Bone Marrow Transplant* 1996; **17**: 191-196.
172. Woods WG, Neudorf S, Gold S, Sanders J, Buckley JD, Barnard DR, Dusenbery K, DeSwarte J, Arthur DC, Lange BJ, Kobrinsky NL; Children's Cancer Group. A comparison of allogeneic bone marrow transplantation, autologous bone marrow transplantation, and aggressive chemotherapy in children with acute myeloid leukemia in remission. *Blood* 2001; **97**: 56-62.
173. Bloomfield CD, Lawrence D, Byrd JC, Carroll A, Pettenati MJ, Tantravahi R, Patil SR, Davey FR, Berg DT, Schiffer CA, Arthur DC, Mayer RJ. Frequency of prolonged remission duration after high-dose cytarabine intensification in acute myeloid leukemia varies by cytogenetic subtype. *Cancer Res* 1998; **58**: 4173-4179.
174. Byrd JC, Dodge RK, Carroll A, Baer MR, Edwards C, Stamberg J, Qumsiyeh M, Moore JO, Mayer RJ, Davey F, Schiffer CA, Bloomfield CD. Patients with t(8;21)(q22;q22) and acute myeloid leukemia have superior failure-free and overall survival when repetitive cycles of high-dose cytarabine are administered. *J Clin Oncol* 1999; **17**: 3767-3775.
175. Perel Y, Auvrignon A, Leblanc T, Vannier JP, Michel G, Nelken B, Gandemer V, Schmitt C, Lamagnere JP, De Lumley L, Bader-Meunier B, Couillaud G, Schaison G, Landman-Parker J, Thuret I, Dalle JH, Baruchel A, Leverger G; Group LAME of the French Society of Pediatric Hematology and Immunology. Impact of addition of maintenance therapy to intensive induction and consolidation chemotherapy for childhood acute myeloblastic leukemia: results of a prospective randomized trial, LAME 89/91. Leucamie Aique Myeloide Enfant. *J Clin Oncol*. 2002; **20**: 2774-2782.
176. Weick JK, Kopecky KJ, Appelbaum FR, Head DR, Kingsbury LL, Balcerzak SP, Bickers JN, Hynes HE, Welborn JL, Simon SR, Grever M. A randomized investigation of high-dose versus standard-dose cytosine arabinoside with daunorubicin in patients with previously untreated acute myeloid leukemia: a Southwest Oncology Group study. *Blood* 1996; **88**: 2841-2851.
177. Mayer RJ, Davis RB, Schiffer CA, Berg DT, Powell BL, Schulman P, Omura GA, Moore JO, McIntyre OR, Frei E 3rd. Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia Group B. *N Engl J Med* 1994; **331**: 896-903.

178. Löwenberg B, Suciu S, Archimbaud E, Haak H, Stryckmans P, De Cataldo R, Dekker AW, Berneman ZN, Thyss A, Van der Lelie J, Sonneveld P, Visani G, Fillet G, Hayat M, Hagemeijer A, Solbu G, Zittoun R. Mitoxantrone versus daunorubicin in induction-consolidation chemotherapy-the value of low-dose cytarabine for maintenance of remission, and an assessment of prognostic factors in acute myeloid leukemia in the elderly: final report. European Organization for the Research and Treatment of Cancer and the Dutch-Belgian Hemato-Oncology Cooperative Hovon Group. *J Clin Oncol* 1998; **16**: 872-881.
179. Swansbury GJ, Lawler SD, Alimena G, Arthur D, Berger R, Van den Berghe H, Bloomfield CD, De la Chappelle A, Dewald G, Garson OM, *et al*. Long-term survival in acute myelogenous leukemia: a second follow-up of the Fourth International Workshop on Chromosomes in Leukemia. *Cancer Genet Cytogenet* 1994; **73**: 1-7.
180. Burnett AK. Acute myeloid leukemia: treatment of adults under 60 years. *Rev Clin Exp Hematol* 2002; **6**: 26-45; discussion 86-87.
181. Haferlach T, Kern W, Schoch C, Schnittger S, Sauerland MC, Heinecke A, Buchner T, Hiddemann W; German AML Cooperative Group. A new prognostic score for patients with acute myeloid leukemia based on cytogenetics and early blast clearance in trials of the German AML Cooperative Group. *Haematologica* 2004; **89**: 408-418.
182. Kern W, Haferlach T, Schoch C, Loffler H, Gassmann W, Heinecke A, Sauerland MC, Berdel W, Buchner T, Hiddemann W. Early blast clearance by remission induction therapy is a major independent prognostic factor for both achievement of complete remission and long-term outcome in acute myeloid leukemia: data from the German AML Cooperative Group (AMLCG) 1992 Trial. *Blood* 2003; **101**: 64-70.
183. Leahey A, Kelly K, Rorke LB, Lange B. A phase I/II study of idarubicin (Ida) with continuous infusion fludarabine (F-ara-A) and cytarabine (ara-C) for refractory or recurrent pediatric acute myeloid leukemia (AML). *J Pediatr Hematol Oncol* 1997; **19**: 304-308.
184. Thomas X, Cambier N, Taksin AL, Reman O, Vekhoff A, Pautas C, Leblond V, Soler-Michel P, Ecstein-Fraisse E, Archimbaud E. Dose-escalation study of single dose mitoxantrone in combination with timed sequential chemotherapy in patients with refractory or relapsing acute myelogenous leukemia. *Leuk Res* 2000; **24**: 957-963.
185. Webb DK, Wheatley K, Harrison G, Stevens RF, Hann IM. Outcome for children with relapsed acute myeloid leukaemia following initial therapy in the Medical Research Council (MRC) AML 10 trial. MRC Childhood Leukaemia Working Party. *Leukemia* 1999; **13**: 25-31.
186. Davies SM, Wagner JE, Shu XO, Blazar BR, Katsanis E, Orchard PJ, Kersey JH, Dusenbery KE, Weisdorf DJ, McGlave PB, Ramsay NK. Unrelated donor bone marrow transplantation for children with acute leukemia. *J Clin Oncol* 1997; **15**: 557-565.
187. Casper J, Camitta B, Truitt R, Baxter-Lowe LA, Bunin N, Lawton C, Murray K, Hunter J, Pietryga D, Garbrecht F, *et al*. Unrelated bone marrow donor transplants for children with leukemia or myelodysplasia. *Blood* 1995; **85**: 2354-2363.
188. Estey EH. Treatment of relapsed and refractory acute myelogenous leukemia. *Leukemia* 2000; **14**: 476-479.
189. Hehlmann R, Heimpel H, Hasford J, Kolb HJ, Pralle H, Hossfeld DK, Queisser W, Loffler H, Heinze B, Georgii A, *et al*. Randomized comparison of busulfan and hydroxyurea in chronic myelogenous leukemia: prolongation of survival by hydroxyurea. The German CML Study Group. *Blood* 1993; **82**: 398-407.
190. Stone RM. Optimizing treatment of chronic myeloid leukemia: a rational approach. *Oncologist* 2004; **9**: 259-270.
191. Kantarjian HM, Giles FJ, O'Brien SM, Talpaz M. Clinical course and therapy of chronic myelogenous leukemia with interferon-alpha and chemotherapy. *Hematol Oncol Clin North Am* 1998; **12**: 31-80.
192. The Italian Cooperative Study Group on Chronic Myeloid Leukemia. Interferon Alfa-2a as Compared with Conventional Chemotherapy for the Treatment of Chronic Myeloid Leukemia. *N Engl J Med* 1994; **330**: 820-825.

193. Guilhot F, Chastang C, Michallet M, Guerci A, Harousseau JL, Maloisel F, Bouabdallah R, Guyotat D, Cheron N, Nicolini F, Abgrall JF, Tanzer J. Interferon alfa-2b combined with cytarabine versus interferon alone in chronic myelogenous leukemia. French Chronic Myeloid Leukemia Study Group. *N Engl J Med* 1997; **337**: 223-229.
194. Clift RA, Storb R. Marrow transplantation for CML: the Seattle experience. *Bone Marrow Transplant* 1996 ;**17**: S1-3.
195. Horowitz MM, Rowlings PA, Passweg JR. Allogeneic bone marrow transplantation for CML: a report from the International Bone Marrow Transplant Registry. *Bone Marrow Transplant* 1996; **17**: S5-6.
196. Van Rhee F, Szydlo RM, Hermans J, Devergie A, Frassoni F, Arcese W, De Witte T, Kolb HJ, Niederwiser D, Jacobsen N, Gahrton G, Bandini G, Carreras E, Bacigalupo A, Michallet M, Ruutu T, Reiffers J, Goldman JM, Apperley J, Gratwohl A. Long-term results after allogeneic bone marrow transplantation for chronic myelogenous leukemia in chronic phase: a report from the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant* 1997; **20**: 553-560.
197. Gratwohl A, Hermans J, Niederwieser D, Frassoni F, Arcese W, Gahrton G, Bandini G, Carreras E, Vernant JP, Bosi A, *et al*. Bone marrow transplantation for chronic myeloid leukemia: long-term results. Chronic Leukemia Working Party of the European Group for Bone Marrow Transplantation. *Bone Marrow Transplant* 1993; **12**: 509-516.
198. Silver RT, Woolf SH, Hehlmann R, Appelbaum FR, Anderson J, Bennett C, Goldman JM, Guilhot F, Kantarjian HM, Lichtin AE, Talpaz M, Tura S. An evidence-based analysis of the effect of busulfan, hydroxyurea, interferon, and allogeneic bone marrow transplantation in treating the chronic phase of chronic myeloid leukemia: developed for the American Society of Hematology. *Blood* 1999; **94**: 1517-1536.
199. Weisdorf DJ, Anasetti C, Antin JH, Kernan NA, Kollman C, Snyder D, Petersdorf E, Nelson G, McGlave P. Allogeneic bone marrow transplantation for chronic myelogenous leukemia: comparative analysis of unrelated versus matched sibling donor transplantation. *Blood* 2002; **99**: 1971-1977.
200. Hansen JA, Gooley TA, Martin PJ, Appelbaum F, Chauncey TR, Clift RA, Petersdorf EW, Radich J, Sanders JE, Storb RF, Sullivan KM, Anasetti C. Bone marrow transplants from unrelated donors for patients with chronic myeloid leukemia. *N Engl J Med* 1998; **338**: 962-968.
201. Or R, Shapira MY, Resnick I, Amar A, Ackerstein A, Samuel S, Aker M, Naparstek E, Nagler A, Slavin S. Nonmyeloablative allogeneic stem cell transplantation for the treatment of chronic myeloid leukemia in first chronic phase. *Blood* 2003; **101**: 441-445.
202. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, Ljungman P, Ferrant A, Verdonck L, Niederwieser D, Van Rhee F, Mittermueller J, De Witte T, Holler E, Ansari H, European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* 1995; **86**: 2041-2050.
203. Porter DL, Antin JH. Infusion of donor peripheral blood mononuclear cells to treat relapse after transplantation for chronic myelogenous leukemia. *Hematol Oncol Clin North Am* 1998; **12**: 123-150.
204. Warrell RP Jr, De The H, Wang ZY, Degos L. Acute promyelocytic leukemia. *N Engl J Med* 1993; **329**: 177-189.
205. Piazza F, Gurrieri C, Pandolfi PP. The theory of APL. *Oncogene* 2001; **20**: 7216-7222.
206. Fenaux P, Chastang C, Chevret S, Sanz M, Dombret H, Archimbaud E, Fey M, Rayon C, Huguet F, Sotto JJ, Gardin C, Makhoul PC, Travade P, Solary E, Fegueux N, Bordessoule D, Miguel JS, Link H, Desablens B, Stamatoullas A, Deconinck E, Maloisel F, Castaigne S, Preudhomme C, Degos L. A randomized comparison of all transretinoic acid (ATRA) followed by chemotherapy and ATRA plus chemotherapy and the role of maintenance therapy in newly diagnosed acute promyelocytic leukemia. The European APL Group. *Blood* 1999; **94**: 1192-1200.

207. Mandelli F, Diverio D, Avvisati G, Luciano A, Barbui T, Bernasconi C, Broccia G, Cerri R, Falda M, Fioritoni G, Leoni F, Liso V, Petti MC, Rodeghiero F, Saglio G, Vegna ML, Visani G, Jehn U, Willemze R, Muus P, Pelicci PG, Biondi A, Lo Coco F. Molecular remission in PML/RAR alpha-positive acute promyelocytic leukemia by combined *all-trans* retinoic acid and idarubicin (AIDA) therapy. Gruppo Italiano-Malattie Ematologiche Maligne dell'Adulto and Associazione Italiana di Ematologia ed Oncologia Pediatrica Cooperative Groups. *Blood* 1997; **90**: 1014-1021.
208. Avvisati G, Petti MC, Lo-Coco F, Vegna ML, Amadori S, Baccarani M, Cantore N, Di Bona E, Ferrara F, Fioritoni G, Gallo E, Invernizzi R, Lazzarino M, Liso V, Mariani G, Ricciuti F, Selleri C, Sica S, Veneri D, Mandelli F; GIMEMA (Gruppo Italiano Malattie Ematologische dell'Adulto) Italian Cooperative Group. Induction therapy with idarubicin alone significantly influences event-free survival duration in patients with newly diagnosed hypergranular acute promyelocytic leukemia: final results of the GIMEMA randomized study LAP 0389 with 7 years of minimal follow-up. *Blood* 2002; **100**: 3141-3146.
209. Estey E, Thall PF, Pierce S, Kantarjian H, Keating M. Treatment of newly diagnosed acute promyelocytic leukemia without cytarabine. *J Clin Oncol* 1997; **15**: 483-490.
210. Ohno R, Asou N; Japan Adult Leukemia Study Group. The recent JALSG study for newly diagnosed patients with acute promyelocytic leukemia (APL). *Ann Hematol* 2004; **83**: S77-78.
211. Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY, Zhu J, Tang W, Sun GL, Yang KQ, Chen Y, Zhou L, Fang ZW, Wang YT, Ma J, Zhang P, Zhang TD, Chen SJ, Chen Z, Wang ZY. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood* 1997; **89**: 3354-3360.
212. Novick SC, Warrell RP Jr. Arsenicals in hematologic cancers. *Semin Oncol* 2000; **27**: 495-501.
213. Soignet SL, Maslak P, Wang ZG, Jhanwar S, Calleja E, Dardashti LJ, Corso D, DeBlasio A, Gabrilove J, Scheinberg DA, Pandolfi PP, Warrell RP Jr. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. *N Engl J Med* 1998; **339**: 1341-1348.
214. De Botton S, Fawaz A, Chevret S, Dombret H, Thomas X, Sanz M, Guerci A, San Miguel J, De la Serna J, Stoppa AM, Reman O, Stamatoulas A, Fey M, Cahn JY, Sotto JJ, Bourhis JH, Parry A, Chomienne C, Degos L, Fenaux P. Autologous and allogeneic stem-cell transplantation as salvage treatment of acute promyelocytic leukemia initially treated with *all-trans*-retinoic acid: a retrospective analysis of the European acute promyelocytic leukemia group. *J Clin Oncol* 2005; **23**: 120-126.
215. Tallman MS, Andersen JW, Schiffer CA, Appelbaum FR, Feusner JH, Ogden A, Shepherd L, Willman C, Bloomfield CD, Rowe JM, Wiernik PH. *All-trans*-Retinoic Acid in Acute Promyelocytic Leukemia. *N Engl J Med* 1997; **337**: 1021-1028.
216. Tallman MS, Andersen JW, Schiffer CA, Appelbaum FR, Feusner JH, Woods WG, Ogden A, Weinstein H, Shepherd L, Willman C, Bloomfield CD, Row JM, Wiernik PH. *All-trans* retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup protocol. *Blood* 2002; **100**: 4298-4302.
217. Gallagher RE. Retinoic acid resistance in acute promyelocytic leukemia. *Leukemia* 2002; **16**: 1940-1958.
218. Zein N, Sinha AM, McGahren WJ, Ellestad GA. Calicheamicin gamma 1I: an antitumor antibiotic that cleaves double-stranded DNA site specifically. *Science* 1988; **240**: 1198-1201.
219. Andrews RG, Torok-Storb B, Bernstein ID. Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. *Blood* 1983; **62**: 124-132.
220. Griffin JD, Linch D, Sabbath K, Larcom P, Schlossman SF. A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. *Leuk Res* 1984; **8**: 521-534.
221. Larson RA, Boogaerts M, Estey E, Karanes C, Stadtmauer EA, Sievers EL, Mineur P, Bennett JM, Berger MS, Eten CB, Munteanu M, Loken MR, Van Dongen JJM, Bernstein ID, Appelbaum FR; Mylotarg Study Group. Antibody-targeted chemotherapy of older patients with acute myeloid leukemia in first relapse using Mylotarg (gemtuzumab ozogamicin). *Leukemia* 2002; **16**: 1627-1636.

222. Sievers EL, Larson RA, Stadtmauer EA, Estey E, Löwenberg B, Dombret H, Karanes C, Theobald M, Bennett JM, Sherman ML, Berger MS, Eten CB, Loken MR, Van Dongen JJM, Bernstein ID, Appelbaum FR; Mylotarg Study Group. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J Clin Oncol* 2001; **19**: 3244-3254.
223. Nabhan C, Rundhaugen LM, Riley MB, Rademaker A, Boehlke L, Jatoi M, Tallman MS. Phase II pilot trial of gemtuzumab ozogamicin (GO) as first line therapy in acute myeloid leukemia patients age 65 or older. *Leuk Res* 2005; **29**: 53-57.
224. Estey EH, Thall PF, Giles FJ, Wang XM, Cortes JE, Beran M, Pierce SA, Thomas DA, Kantarjian HM. Gemtuzumab ozogamicin with or without interleukin 11 in patients 65 years of age or older with untreated acute myeloid leukemia and high-risk myelodysplastic syndrome: comparison with idarubicin plus continuous-infusion, high-dose cytosine arabinoside. *Blood* 2002; **99**: 4343-4349.
225. Piccaluga PP, Martinelli G, Rondoni M, Malagola M, Gaitani S, Visani G, Baccarani M. First experience with gemtuzumab ozogamicin plus cytarabine as continuous infusion for elderly acute myeloid leukaemia patients. *Leuk Res* 2004; **28**: 987-990.
226. Amadori S, Suciu S, Willemze R, Mandelli F, Selleslag D, Stauder R, Ho A, Denzlinger C, Leone G, Fabris P, Muus P, Vignetti M, Hagemeijer A, Beeldens F, Anak O, De Witte T; EORTC leukemia group; GIMEMA leukemia group. Sequential administration of gemtuzumab ozogamicin and conventional chemotherapy as first line therapy in elderly patients with acute myeloid leukemia: a phase II study (AML-15) of the EORTC and GIMEMA leukemia groups. *Haematologica* 2004; **89**: 950-956.
227. Reinhardt D, Diekamp S, Fleischhack G, Corbacioglu C, Jurgens H, Dworzak M, Kaspers G, Creutzig U, Zwaan CM. Gemtuzumab ozogamicin (Mylotarg) in children with refractory or relapsed acute myeloid leukemia. *Onkologie* 2004; **27**: 269-272.
228. Zwaan CM, Reinhardt D, Corbacioglu S, Van Wering ER, Bokkerink JP, Tissing WJ, Samuelsson U, Feingold J, Creutzig U, Kaspers GJ. Gemtuzumab ozogamicin: first clinical experiences in children with relapsed/refractory acute myeloid leukemia treated on compassionate-use basis. *Blood* 2003; **101**: 3868-3871.
229. Petti MC, Pinazzi MB, Diverio D, Romano A, Petrucci MT, De Santis S, Meloni G, Tafuri A, Mandelli F, Lo Coco F. Prolonged molecular remission in advanced acute promyelocytic leukaemia after treatment with gemtuzumab ozogamicin (Mylotarg CMA-676). *Br J Haematol* 2001; **115**: 63-65.
230. Lo-Coco F, Cimino G, Breccia M, Noguera NI, Diverio D, Finolezzi E, Pogliani EM, Di Bona E, Micalizzi C, Kropp M, Venditti A, Tafuri A, Mandelli F. Gemtuzumab ozogamicin (Mylotarg) as a single agent for molecularly relapsed acute promyelocytic leukemia. *Blood* 2004; **104**: 1995-1999.
231. Estey EH, Giles FJ, Beran M, O'Brien S, Pierce SA, Faderl SH, Cortes JE, Kantarjian HM. Experience with gemtuzumab ozogamycin ("mylotarg") and all-trans retinoic acid in untreated acute promyelocytic leukemia. *Blood* 2002; **99**: 4222-4224.
232. Kell WJ, Burnett AK, Chopra R, Yin JA, Clark RE, Rohatiner A, Culligan D, Hunter A, Prentice AG, Milligan DW. A feasibility study of simultaneous administration of gemtuzumab ozogamicin with intensive chemotherapy in induction and consolidation in younger patients with acute myeloid leukemia. *Blood* 2003; **102**: 4277-4283.
233. Walter RB, Raden BW, Hong TC, Flowers DA, Bernstein ID, Linenberger ML. Multidrug resistance protein attenuates gemtuzumab ozogamicin-induced cytotoxicity in acute myeloid leukemia cells. *Blood* 2003; **102**: 1466-1473.
234. Matsui H, Takeshita A, Naito K, Shinjo K, Shigeno K, Maekawa M, Yamakawa Y, Tanimoto M, Kobayashi M, Ohnishi K, Ohno R. Reduced effect of gemtuzumab ozogamicin (CMA-676) on P-glycoprotein and/or CD34-positive leukemia cells and its restoration by multidrug resistance modifiers. *Leukemia* 2002; **16**: 813-819.

235. Van der Velden VHJ, Boeckx N, Jedema I, Te Marvelde JG, Hoogeveen PG, Boogaerts M, Van Dongen JJM. High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg) treatment in acute myeloid leukemia patients. *Leukemia* 2004; **18**: 983-988.
236. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. Efficacy and safety of a specific inhibitor of the *BCR-ABL* tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001; **344**: 1031-1037.
237. Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, Niederwieser D, Resta D, Capdeville R, Zoellner U, Talpaz M, Druker B, Goldman J, O'Brien SG, Russell N, Fischer T, Ottmann O, Cony-Makhoul P, Facon T, Stone R, Miller C, Tallman M, Brown R, Schuster M, Loughran T, Gratwohl A, Mandelli F, Saglio G, Lazzarino M, Russo D, Baccarani M, Morra E; International STI571 CML Study Group. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med* 2002; **346**: 645-652.
238. Talpaz M, Silver RT, Druker BJ, Goldman JM, Gambacorti-Passerini C, Guilhot F, Schiffer CA, Fischer T, Deininger MW, Lennard AL, Hochhaus A, Ottmann OG, Gratwohl A, Baccarani M, Stone R, Tura S, Mahon FX, Fernandes-Reese S, Gathmann I, Capdeville R, Kantarjian HM, Sawyers CL. Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood* 2002; **99**: 1928-1937.
239. Sawyers CL, Hochhaus A, Feldman E, Goldman JM, Miller CB, Ottmann OG, Schiffer CA, Talpaz M, Guilhot F, Deininger MW, Fischer T, O'Brien SG, Stone RM, Gambacorti-Passerini CB, Russell NH, Reiffers JJ, Shea TC, Chapuis B, Coutre S, Tura S, Morra E, Larson RA, Saven A, Peschel C, Gratwohl A, Mandelli F, Ben-Am M, Gathmann I, Capdeville R, Paquette RL, Druker BJ. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* 2002; **99**: 3530-3539.
240. O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, Cornelissen JJ, Fischer T, Hochhaus A, Hughes T, Lechner K, Nielsen JL, Rousselot P, Reiffers J, Saglio G, Shepherd J, Simonsson B, Gratwohl A, Goldman JM, Kantarjian H, Taylor K, Verhoef G, Bolton AE, Capdeville R, Druker BJ; IRIS Investigators. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 2003; **348**: 994-1004.
241. Hughes TP, Kaeda J, Branford S, Rudzki Z, Hochhaus A, Hensley ML, Gathmann I, Bolton AE, Van Hoornissen IC, Goldman JM, Radich JP; International Randomised Study of Interferon versus STI571 (IRIS) Study Group. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. *N Engl J Med* 2003; **349**: 1423-1432.
242. Guilhot F. Sustained Durability of Responses Plus High Rates of Cytogenetic Responses Result in Long-Term Benefit for Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia (CML-CP) Treated with Imatinib (IM) Therapy: Update from the IRIS Study. *Blood* 2004; **104** (abstract 21).
243. Fischer T, Reifenrath C, Hess GR, Corsetti MT, Kreil S, Beck J, Meinhardt P, Beltrami G, Schuch B, Gschaidmeier H, Hehlmann R, Hochhaus A, Carella A, Huber C. Safety and efficacy of STI-571 (imatinib mesylate) in patients with *BCR/ABL*-positive chronic myelogenous leukemia (CML) after autologous peripheral blood stem cell transplantation (PBSCT). *Leukemia* 2002; **16**: 1220-1228.
244. Olavarria E, Ottmann OG, Deininger M, Clark RE, Bandini G, Byrne J, Lipton J, Vitek A, Michallet M, Siegert W, Ullmann A, Wassmann B, Niederwieser D, Fischer T; Chronic Leukaemia Working Party of the European Group of Bone and Marrow Transplantation (EBMT). Response to imatinib in patients who relapse after allogeneic stem cell transplantation for chronic myeloid leukemia. *Leukemia* 2003; **17**: 1707-1712.

245. Deininger MW, O'Brien SG, Ford JM, Druker BJ. Practical management of patients with chronic myeloid leukemia receiving imatinib. *J Clin Oncol* 2003; **21**: 1637-1647.
246. Stone RM. Optimizing treatment of chronic myeloid leukemia: a rational approach. *Oncologist* 2004; **9**: 259-270.
247. Roche-Lestienne C, Soenen-Cornu V, Grardel-Duflos N, Lai JL, Philippe N, Facon T, Fenaux P, Preudhomme C. Several types of mutations of the *ABL* gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment. *Blood* 2002; **100**: 1014-1018.
248. Kreuzer KA, Le Coutre P, Landt O, Na IK, Schwarz M, Schultheis K, Hochhaus A, Dorken B. Preexistence and evolution of imatinib mesylate-resistant clones in chronic myelogenous leukemia detected by a PNA-based PCR clamping technique. *Ann Hematol* 2003; **82**: 284-289.
249. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL. Clinical resistance to STI-571 cancer therapy caused by *BCR-ABL* gene mutation or amplification. *Science* 2001; **293**: 876-880.
250. Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, Herrmann R, Lynch KP, Hughes TP. High frequency of point mutations clustered within the adenosine triphosphate-binding region of *BCR/ABL* in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. *Blood* 2002; **99**: 3472-3475.
251. Le Coutre P, Tassi E, Varella-Garcia M, Barni R, Mologni L, Cabrita G, Marchesi E, Supino R, Gambacorti-Passerini C. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood* 2000; **95**: 1758-1766.
252. Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, Sawyers CL. Multiple *BCR-ABL* kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* 2002; **2**: 117-125.
253. Hochhaus A, La Rosee P. Imatinib therapy in chronic myelogenous leukemia: strategies to avoid and overcome resistance. *Leukemia* 2004; **18**: 1321-1331.
254. Hochhaus A, Kreil S, Corbin AS, La Rosee P, Muller MC, Lahaye T, Hanfstein B, Schoch C, Cross NC, Berger U, Gschaidmeier H, Druker BJ, Hehlmann R. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. *Leukemia* 2002; **16**: 2190-2196.
255. Branford S, Rudzki Z, Walsh S, Parkinson I, Grigg A, Szer J, Taylor K, Herrmann R, Seymour JF, Arthur C, Joske D, Lynch K, Hughes T. Detection of *BCR-ABL* mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood* 2003; **102**: 276-283.
256. Corbin AS, La Rosee P, Stoffregen EP, Druker BJ, Deininger MW. Several *BCR-ABL* kinase domain mutants associated with imatinib mesylate resistance remain sensitive to imatinib. *Blood* 2003; **101**: 4611-4614.
257. Deininger MW, Druker BJ. Specific targeted therapy of chronic myelogenous leukemia with imatinib. *Pharmacol Rev* 2003; **55**: 401-423.
258. Yamamoto M, Kurosu T, Kakihana K, Mizuchi D, Miura O. The two major imatinib resistance mutations E255K and T315I enhance the activity of BCR/ABL fusion kinase. *Biochem Biophys Res Commun* 2004; **319**: 1272-1275.
259. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* 2004; **305**: 399-401.
260. Shah NP, Branford S, Hughes TP, Nicoll JM, Decillis AP, Sawyers CL. Major Cytogenetic Responses to BMS-354825 in Patients with Chronic Myeloid Leukemia Are Associated with a One to Two Log Reduction in *BCR-ABL* Transcript. *Blood* 2004; **104** (abstract 1008).

261. Giles F, Kantarjian H, Wassmann B, Cortes J, O'Brien S, Tanaka C, Rae P, Mietlowski W, Romano A, Alland L, Dugan M, Albitar M, Ottmann O. Phase I/II Study of AMN107, a Novel Aminopyrimidine Inhibitor of *BCR-ABL*, on a Continuous Daily Dosing Schedule in Adult Patients (pts) with Imatinib-Resistant Advanced Phase Chronic Myeloid Leukemia (CML) or Relapsed/ Refractory Philadelphia Chromosome (Ph+) Acute Lymphocytic Leukemia (ALL). *Blood* 2004; **104** (abstract 22).
262. Talpaz M, Kantarjian H, Shah NP, Donato N, Nicoll J, Bai SA, Huang F, Clark E, DeCillis AP, Sawyers C. Hematologic and Cytogenetic Responses in Imatinib-Resistant Accelerated and Blast Phase Chronic Myeloid Leukemia (CML) Patients Treated with the Dual SRC/ABL Kinase Inhibitor BMS-354825: Results from a Phase I Dose Escalation Study. *Blood* 2004; **104** (abstract 20).
263. Mestan J, Weisberg E, Cowan-Jacob S, Fabbro D, Furet P, Fendrich G, Goutte Ge, Kempf D, Gaugler M, Griffin DJ, Manley WP. AMN107: *In vitro* profile of a new inhibitor of the tyrosine kinase activity of *BCR-ABL*. *Blood* 2004; **104** (abstract 1978).
264. Ahmad K. New agent overcomes resistance to imatinib. *Lancet Oncol* 2005; **6**: 137.
265. Gumireddy K, Baker SJ, Cosenza SC, John P, Kang AD, Robell KA, Reddy MV, Reddy EP. A non-ATP-competitive inhibitor of *BCR-ABL* overrides imatinib resistance. *Proc Natl Acad Sci USA* 2005; **102**: 1992-1997.
266. Harousseau JL, Cahn JY, Pignon B, Witz F, Milpied N, Delain M, Lioure B, Lamy T, Desablens B, Guilhot F, Caillot D, Abgrall JF, Francois S, Briere J, Guyotat D, Casassus P, Audhuy B, Tellier Z, Hurteloup P, Herve P. Comparison of autologous bone marrow transplantation and intensive chemotherapy as postremission therapy in adult acute myeloid leukemia. The Groupe Ouest Est Leucemies Aigues Myeloblastiques (GOELAM). *Blood* 1997; **90**: 2978-2986.
267. Heil G, Hoelzer D, Sanz MA, Lechner K, Liu Yin JA, Papa G, Noens L, Szer J, Ganser A, O'Brien C, Matcham J, Barge A. A randomized, double-blind, placebo-controlled, phase III study of filgrastim in remission induction and consolidation therapy for adults with *de novo* acute myeloid leukemia. The International Acute Myeloid Leukemia Study Group. *Blood* 1997; **90**: 4710-4718.
268. Cave H, Van der Werff ten Bosch J, Suciu S, Guidal C, Waterkeyn C, Otten J, Bakkus M, Thielemans K, Grandchamp B, Vilmer E. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer-Childhood Leukemia Cooperative Group. *N Engl J Med* 1998; **339**: 591-598.
269. Van Dongen JJM, Seriu T, Panzer-Grumayer ER, Biondi A, Pongers-Willemse MJ, Corral L, Stolz F, Schrappe M, Masera G, Kamps WA, Gadner H, Van Wering ER, Ludwig WD, Basso G, De Bruijn MA, Cazzaniga G, Hettinger K, Van der Does-van den Berg A, Hop WC, Riehm H, Bartram CR. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. *Lancet* 1998; **352**: 1731-1738.
270. Mortuza FY, Papaioannou M, Moreira IM, Coyle LA, Gameiro P, Gandini D, Prentice HG, Goldstone A, Hoffbrand AV, Foroni L. Minimal residual disease tests provide an independent predictor of clinical outcome in adult acute lymphoblastic leukemia. *J Clin Oncol* 2002; **20**: 1094-1104.
271. Bruggemann M, Raff T, Flohr T, Gokbuget N, Nakao M, Droese J, Luschen S, Pott C, Ritgen M, Scheuring U, Horst HA, Thiel E, Hoelzer D, Bartram CR, Kneba M. Clinical significance of minimal residual disease quantification in adult patients with standard risk acute lymphoblastic leukemia. *Blood* 2006; **107**: 1116-1123.
272. Lo Coco F, Diverio D, Avvisati G, Petti MC, Meloni G, Pogliani EM, Biondi A, Rossi G, Carlo-Stella C. Selleri C, Martino B, Specchia G, Mandelli F. Therapy of molecular relapse in acute promyelocytic leukemia. *Blood* 1999; **94**: 2225-2229.
273. Oehler VG, Radich JP. Monitoring *BCR-ABL* by polymerase chain reaction in the treatment of chronic myeloid leukemia. *Curr Oncol Rep* 2003; **5**: 426-435.
274. Roman J, Alvarez MA, Torres A. Molecular basis for therapeutic decisions in chronic myeloid leukemia patients after allogeneic bone marrow transplantation. *Haematologica* 2000; **85**: 1072-1082.

275. San Miguel JF, Vidriales MB, Lopez-Berges C, Diaz-Mediavilla J, Gutierrez N, Canizo C, Ramos F, Calmuntia MJ, Perez JJ, Gonzalez M, Orfao A. Early immunophenotypical evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups and may contribute to postinduction treatment stratification. *Blood* 2001; **98**: 1746-1751.
276. Coustan-Smith E, Ribeiro RC, Rubnitz JE, Razzouk BI, Pui CH, Pounds S, Andreansky M, Behm FG, Raimondi SC, Shurtleff SA, Downing JR, Campana D. Clinical significance of residual disease during treatment in childhood acute myeloid leukaemia. *Br J Haematol* 2003; **123**: 243-252.
277. San Miguel JF, Vidriales MB, Orfao A. Immunological evaluation of minimal residual disease (MRD) in acute myeloid leukaemia (AML). *Best Pract Res Clin Haematol* 2002; **15**: 105-118.
278. Macedo A, Orfao A, Ciudad J, Gonzalez M, Vidriales B, Lopez-Berges MC, Martinez A, Landolfi C, Canizo C, San Miguel JF. Phenotypic analysis of CD34 subpopulations in normal human bone marrow and its application for the detection of minimal residual disease. *Leukemia* 1995; **9**: 1896-1901.
279. Langebrake C, Brinkmann I, Teigler-Schlegel A, Creutzig U, Griesinger F, Puhlmann U, Reinhardt D. Immunophenotypic differences between diagnosis and relapse in childhood AML: Implications for MRD monitoring. *Cytometry B Clin Cytom* 2004; **63**: 1-9.
280. Macedo A, San Miguel JF, Vidriales MB, Lopez-Berges MC, Garcia-Marcos MA, Gonzalez M, Landolfi C, Orfao A. Phenotypic changes in acute myeloid leukaemia: implications in the detection of minimal residual disease. *J Clin Pathol* 1996; **49**: 15-18.
281. Baer MR, Stewart CC, Dodge RK, Leget G, Sule N, Mrozek K, Schiffer CA, Powell BL, Kolitz JE, Moore JO, Stone RM, Davey FR, Carroll AJ, Larson RA, Bloomfield CD. High frequency of immunophenotype changes in acute myeloid leukemia at relapse: implications for residual disease detection (Cancer and Leukemia Group B Study 8361). *Blood* 2001; **97**: 3574-3580.
282. Hur M, Chang YH, Lee DS, Park MH, Cho HI. Immunophenotypic and cytogenetic changes in acute leukaemia at relapse. *Clin Lab Haematol* 2001; **23**: 173-179.
283. Tomova A, Babusikova O. Shifts in expression of immunological cell markers in relapsed acute leukemia. *Neoplasma* 2001; **48**: 164-168.
284. Thomas X, Campos L, Archimbaud E, Shi ZH, Treille-Ritouet D, Anglaret B, Fiere D. Surface marker expression in acute myeloid leukaemia at first relapse. *Br J Haematol* 1992; **81**: 40-44.
285. Gabert J, Beillard E, Van der Velden VHJ, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cave H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, Gonzalez M, Viehmann S, Malec M, Saglio G, Van Dongen JJM. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. *Leukemia* 2003; **17**: 2318-2357.
286. Beillard E, Pallisgaard N, Van der Velden VHJ, Bi W, Dee R, Van der Schoot E, Delabesse E, Macintyre E, Gottardi E, Saglio G, Watzinger F, Lion T, Van Dongen JJM, Hokland P, Gabert J. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. *Leukemia* 2003; **17**: 2474-2486.
287. Muller MC, Hordt T, Paschka P, Merx K, La Rosee P, Hehlmann R, Hochhaus A. Standardization of preanalytical factors for minimal residual disease analysis in chronic myelogenous leukemia. *Acta Haematol* 2004; **112**: 30-33.
288. Van der Velden VHJ, Boeckx N, Gonzalez M, Malec M, Barbany G, Lion T, Gottardi E, Pallisgaard N, Beillard E, Hop WC, Hoogeveen PG, Gabert J, Van Dongen JJM; Europe Against Cancer Program. Differential stability of control gene and fusion gene transcripts over time may hamper accurate quantification of minimal residual disease-a study within the Europe Against Cancer Program. *Leukemia* 2004; **18**: 884-886.

289. Tanner MA, Berk LS, Felten DL, Blidy AD, Bit SL, Ruff DW. Substantial changes in gene expression level due to the storage temperature and storage duration of human whole blood. *Clin Lab Haematol* 2002; **24**: 337-341.
290. Boeckx N, Jansen MWJ, Haskovec C, Vandenberghe P, Van der Velden VHJ, Van Dongen JJM. Identification of e19a2 *BCR-ABL* fusions (*μ*-BCR breakpoints) at the DNA level by ligation-mediated PCR. *Leukemia* 2005; **19**: 1292-1295.
291. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. *Clin Chim Acta* 1991; **198**: 1-91.
292. Van der Velden VHJ, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, Van Dongen JJM. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* 2003; **17**: 1013-1034.
293. Szczepanski T, Willemse MJ, Brinkhof B, Van Wering ER, Van der Burg M, Van Dongen JJM. Comparative analysis of Ig and TCR gene rearrangements at diagnosis and at relapse of childhood precursor-B-ALL provides improved strategies for selection of stable PCR targets for monitoring of minimal residual disease. *Blood* 2002; **99**: 2315-2323.
294. Nakano Y, Kiyoi H, Miyawaki S, Asou N, Ohno R, Saito H, Naoe T. Molecular evolution of acute myeloid leukaemia in relapse: unstable *N-RAS* and *FLT3* genes compared with p53 gene. *Br J Haematol* 1999; **104**: 659-664.
295. Kottaridis PD, Gale RE, Langabeer SE, Frew ME, Bowen DT, Linch DC. Studies of *FLT3* mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of *FLT3* mutations in leukemogenesis, minimal residual disease detection, and possible therapy with *FLT3* inhibitors. *Blood* 2002; **100**: 2393-2398.
296. Hovland R, Gjertsen BT, Bruserud O. Acute myelogenous leukemia with internal tandem duplication of the *FLT3* gene appearing or altering at the time of relapse: a report of two cases. *Leuk Lymphoma* 2002; **43**: 2027-2029.
297. Shih LY, Huang CF, Wu JH, Lin TL, Dunn P, Wang PN, Kuo MC, Lai CL, Hsu HC. Internal tandem duplication of *FLT3* in relapsed acute myeloid leukemia: a comparative analysis of bone marrow samples from 108 adult patients at diagnosis and relapse. *Blood* 2002; **100**: 2387-2392.
298. Schnittger S, Schoch C, Kern W, Hiddemann W, Haferlach T. *FLT3* length mutations as marker for follow-up studies in acute myeloid leukaemia. *Acta Haematol* 2004; **112**: 68-78.
299. Shih LY, Huang CF, Wu JH, Wang PN, Lin TL, Dunn P, Chou MC, Kuo MC, Tang CC. Heterogeneous patterns of *FLT3* Asp(835) mutations in relapsed *de novo* acute myeloid leukemia: a comparative analysis of 120 paired diagnostic and relapse bone marrow samples. *Clin Cancer Res* 2004; **10**: 1326-1332.
300. Ostergaard M, Olesen LH, Hasle H, Kjeldsen E, Hokland P. *WT1* gene expression: an excellent tool for monitoring minimal residual disease in 70% of acute myeloid leukaemia patients - results from a single-centre study. *Br J Haematol* 2004; **125**: 590-600.
301. Tamaki H, Mishima M, Kawakami M, Tsuboi A, Kim EH, Hosen N, Ikegame K, Murakami M, Fujioka T, Masuda T, Taniguchi Y, Nishida S, Osumi K, Soma T, Oji Y, Oka Y, Kawase I, Sugiyama H, Ogawa H. Monitoring minimal residual disease in leukemia using real-time quantitative polymerase chain reaction for Wilms tumor gene (*WT1*). *Int J Hematol* 2003; **78**: 349-356.
302. Cilloni D, Gottardi E, De Micheli D, Serra A, Volpe G, Messa F, Rege-Cambrin G, Guerrasio A, Divona M, Lo Coco F, Saglio G. Quantitative assessment of *WT1* expression by real time quantitative PCR may be a useful tool for monitoring minimal residual disease in acute leukemia patients. *Leukemia* 2002; **16**: 2115-2121.
303. Hosen N, Sonoda Y, Oji Y, Kimura T, Minamiguchi H, Tamaki H, Kawakami M, Asada M, Kanato K, Motomura M, Murakami M, Fujioka T, Masuda T, Kim EH, Tsuboi A, Oka Y, Soma T, Ogawa H, Sugiyama H. Very low frequencies of human normal $CD34^+$ haematopoietic progenitor cells express the Wilms' tumour gene *WT1* at levels similar to those in leukaemia cells. *Br J Haematol* 2002; **116**: 409-420.

304. Maurer U, Weidmann E, Karakas T, Hoelzer D, Bergmann L. Wilms tumor gene (*WT1*) mRNA is equally expressed in blast cells from acute myeloid leukemia and normal $CD34^+$ progenitors. *Blood* 1997; **90**: 4230-4232.
305. Baird PN, Simmons PJ. Expression of the Wilms' tumor gene (*WT1*) in normal hemopoiesis. *Exp Hematol* 1997; **25**: 312-320.
306. Sievers EL, Lange BJ, Buckley JD, Smith FO, Wells DA, Daigneault-Creech CA, Shults KE, Bernstein ID, Loken MR. Prediction of relapse of pediatric acute myeloid leukemia by use of multidimensional flow cytometry. *J Natl Cancer Inst* 1996; **88**: 1483-1488.
307. Sievers EL, Lange BJ, Alonzo TA, Gerbing RB, Bernstein ID, Smith FO, Arceci RJ, Woods WG, Loken MR. Immunophenotypic evidence of leukemia after induction therapy predicts relapse: results from a prospective Children's Cancer Group study of 252 patients with acute myeloid leukemia. *Blood* 2003; **101**: 3398-3406.
308. Venditti A, Buccisano F, Del Poeta G, Maurillo L, Tamburini A, Cox C, Battaglia A, Catalano G, Del Moro B, Cudillo L, Postorino M, Masi M, Amadori S. Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. *Blood* 2000; **96**: 3948-3952.
309. San Miguel JF, Martinez A, Macedo A, Vidriales MB, Lopez-Berges C, Gonzalez M, Caballero D, Garcia-Marcos MA, Ramos F, Fernandez-Calvo J, Calmuntia MJ, Diaz-Mediavilla J, Orfao A. Immunophenotyping investigation of minimal residual disease is a useful approach for predicting relapse in acute myeloid leukemia patients. *Blood* 1997; **90**: 2465-2470.
310. Venditti A, Tamburini A, Buccisano F, Del Poeta G, Maurillo L, Panetta P, Scornajenghi KA, Cox C, Amadori S. Clinical relevance of minimal residual disease detection in adult acute myeloid leukemia. *J Hematother Stem Cell Res* 2002; **11**: 349-357.
311. Feller N, Van der Pol MA, Van Stijn A, Weijers GW, Westra AH, Evertse BW, Ossenkoppele GJ, Schuurhuis GJ. MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. *Leukemia* 2004; **18**: 1380-1390.
312. Viehmann S, Teigler-Schlegel A, Bruch J, Langebrake C, Reinhardt D, Harbott J. Monitoring of minimal residual disease (MRD) by real-time quantitative reverse transcription PCR (RQ-RT-PCR) in childhood acute myeloid leukemia with AML1/ETO rearrangement. *Leukemia* 2003; **17**: 1130-1136.
313. Buonamici S, Ottaviani E, Testoni N, Montefusco V, Visani G, Bonifazi F, Amabile M, Terragna C, Ruggeri D, Piccaluga PP, Isodori A, Malagola M, Baccarani M, Tura S, Martinelli G. Real-time quantitation of minimal residual disease in inv(16)-positive acute myeloid leukemia may indicate risk for clinical relapse and may identify patients in a curable state. *Blood* 2002; **99**: 443-449.
314. Laczika K, Mitterbauer G, Mitterbauer M, Knobl P, Schwarzinger I, Greinix HT, Rabitsch W, Fonatsch C, Mannhalter C, Lechner K, Jaeger U. Prospective monitoring of minimal residual disease in acute myeloid leukemia with inversion(16) by CBFbeta/MYH11 RT-PCR: implications for a monitoring schedule and for treatment decisions. *Leuk Lymphoma* 2001; **42**: 923-931.
315. Mitterbauer G, Zimmer C, Pirc-Danoewinata H, Haas OA, Hojas S, Schwarzinger I, Greinix H, Jager U, Lechner K, Mannhalter C. Monitoring of minimal residual disease in patients with *MLL-AF6*-positive acute myeloid leukaemia by reverse transcriptase polymerase chain reaction. *Br J Haematol* 2000; **109**: 622-628.
316. Morschhauser F, Cayuela JM, Martini S, Baruchel A, Rousselot P, Socie G, Berthou P, Jouet JP, Straetmans N, Sigaux F, Fenaux P, Preudhomme C. Evaluation of minimal residual disease using reverse-transcription polymerase chain reaction in t(8;21) acute myeloid leukemia: a multicenter study of 51 patients. *J Clin Oncol* 2000; **18**: 788-794.
317. Tobal K, Newton J, Macheta M, Chang J, Morgenstern G, Evans PA, Morgan G, Lucas GS, Liu Yin JA. Molecular quantitation of minimal residual disease in acute myeloid leukemia with t(8;21) can identify patients in durable remission and predict clinical relapse. *Blood* 2000; **95**: 815-819.

318. Marcucci G, Caligiuri MA, Dohner H, Archer KJ, Schlenk RF, Dohner K, Maghraby EA, Bloomfield CD. Quantification of *CBFβ/MYH11* fusion transcript by real time RT-PCR in patients with inv (16) acute myeloid leukemia. *Leukemia* 2001; **15**: 1072-1080.
319. Marcucci G, Livak KJ, Bi W, Strout MP, Bloomfield CD, Caligiuri MA. Detection of minimal residual disease in patients with *AML1/ETO*-associated acute myeloid leukemia using a novel quantitative reverse transcription polymerase chain reaction assay. *Leukemia* 1998; **12**: 1482-1489.
320. Laczika K, Novak M, Hilgarth B, Mitterbauer M, Mitterbauer G, Scheidel-Petrovic A, Scholten C, Thalhammer-Scherrer R, Brugger S, Keil F, Schwarzinger I, Haas OA, Lechner K, Jaeger U. Competitive *CBFβ/MYH11* reverse-transcriptase polymerase chain reaction for quantitative assessment of minimal residual disease during postremission therapy in acute myeloid leukemia with inversion(16): a pilot study. *J Clin Oncol* 1998; **16**: 1519-1525.
321. Costello R, Sainty D, Blaise D, Gastaut JA, Gabert J, Poirel H, Buzyn-Veil A, Macintyre E. Prognosis value of residual disease monitoring by polymerase chain reaction in patients with *CBFβ/MYH11*-positive acute myeloblastic leukemia. *Blood* 1997; **89**: 2222-2223.
322. Schnittger S, Weisser M, Schoch C, Hiddemann W, Haferlach T, Kern W. New score predicting for prognosis in *PML-RARA*+, *AML1-ETO*+, or *CBFB-MYH11*+ acute myeloid leukemia based on quantification of fusion transcripts. *Blood* 2003; **102**: 2746-2755.
323. Leroy H, De Botton S, Grardel-Duflos N, Darre S, Leleu X, Roumier C, Morschhauser F, Lai JL, Bauters F, Fenaux P, Preudhomme C. Prognostic value of real-time quantitative PCR (RQ-PCR) in AML with t(8;21). *Leukemia* 2005; **19**: 367-372.
324. Burnett AK, Grimwade D, Solomon E, Wheatley K, Goldstone AH. Presenting white blood cell count and kinetics of molecular remission predict prognosis in acute promyelocytic leukemia treated with all-trans retinoic acid: result of the Randomized MRC Trial. *Blood* 1999; **93**: 4131-4143.
325. Sanz MA, Martin G, Rayon C, Esteve J, Gonzalez M, Diaz-Mediavilla J, Bolufer P, Barragan E, Terol MJ, Gonzalez JD, Colomer D, Chillon C, Rivas C, Gomez T, Ribera JM, Bornstein R, Roman J, Calasanz MJ, Arias J, Alvarez C, Ramos F, Deben G. A modified AIDA protocol with anthracycline-based consolidation results in high antileukemic efficacy and reduced toxicity in newly diagnosed *PML/RAR*alpha-positive acute promyelocytic leukemia. PETHEMA group. *Blood* 1999; **94**: 3015-3021.
326. Diverio D, Rossi V, Avvisati G, De Santis S, Pistilli A, Pane F, Saglio G, Martinelli G, Petti MC, Santoro A, Pelicci PG, Mandelli F, Biondi A, Lo Coco F. Early detection of relapse by prospective reverse transcriptase-polymerase chain reaction analysis of the *PML/RAR*alpha fusion gene in patients with acute promyelocytic leukemia enrolled in the GIMEMA-AIEOP multicenter "AIDA" trial. GIMEMA-AIEOP Multicenter "AIDA" Trial. *Blood* 1998; **92**: 784-789.
327. Lo-Coco F, Breccia M, Diverio D. The importance of molecular monitoring in acute promyelocytic leukaemia. *Best Pract Res Clin Haematol* 2003; **16**: 503-520.
328. Grimwade D, Lo Coco F. Acute promyelocytic leukemia: a model for the role of molecular diagnosis and residual disease monitoring in directing treatment approach in acute myeloid leukemia. *Leukemia* 2002; **16**: 1959-1973.
329. Meloni G, Diverio D, Vignetti M, Avvisati G, Capria S, Petti MC, Mandelli F, Lo Coco F. Autologous bone marrow transplantation for acute promyelocytic leukemia in second remission: prognostic relevance of pretransplant minimal residual disease assessment by reverse-transcription polymerase chain reaction of the *PML/RAR*alpha fusion gene. *Blood* 1997; **90**: 1321-1325.
330. Roman J, Martin C, Torres A, Jimenez MA, Andres P, Flores R, De la Torre MJ, Sanchez J, Serrano J, Falcon M. Absence of detectable *PML-RAR*alpha fusion transcripts in long-term remission patients after BMT for acute promyelocytic leukemia. *Bone Marrow Transplant* 1997; **19**: 679-683.
331. Radich JP, Gehly G, Gooley T, Bryant E, Clift RA, Collins S, Edmands S, Kirk J, Lee A, Kessler P, *et al*. Polymerase chain reaction detection of the *BCR-ABL* fusion transcript after allogeneic marrow transplantation for chronic myeloid leukemia: results and implications in 346 patients. *Blood* 1995; **85**: 2632-2638.

332. Olavarria E, Kanfer E, Szydlo R, Kaeda J, Rezvani K, Cwynarski K, Pocock C, Dazzi F, Craddock C, Apperley JF, Cross NC, Goldman JM. Early detection of *BCR-ABL* transcripts by quantitative reverse transcriptase-polymerase chain reaction predicts outcome after allogeneic stem cell transplantation for chronic myeloid leukemia. *Blood* 2001; **97**: 1560-1565.
333. Mughal TI, Yong A, Szydlo RM, Dazzi F, Olavarria E, Van Rhee F, Kaeda J, Cross NC, Craddock C, Kanfer E, Apperley J, Goldman JM. Molecular studies in patients with chronic myeloid leukaemia in remission 5 years after allogeneic stem cell transplant define the risk of subsequent relapse. *Br J Haematol* 2001; **115**: 569-574.
334. Lange T, Deininger M, Brand R, Hegenbart U, Al-Ali H, Krahl R, Poenisch W, Uharek L, Leiblein S, Gentilini C, Petersdorf E, Storb RF, Niederwieser D. *BCR-ABL* transcripts are early predictors for hematological relapse in chronic myeloid leukemia after hematopoietic cell transplantation with reduced intensity conditioning. *Leukemia* 2004; **18**: 1468-1475.

PART 2

Fusion genes and immunoglobulin and T-cell receptor gene rearrangements as minimal residual disease targets in myeloid leukemias

CHAPTER 2.1

Fusion genes and junctional regions of rearranged immunoglobulin/T-cell receptor genes as PCR-targets for detection of minimal residual disease

Fusion genes result from chromosome aberrations such as translocations, inversions or deletions. In most leukemias the fusion genes are transcribed into fusion gene transcripts which are frequently similar between individual patients despite distinct breakpoints at the DNA level (Figure 1). After reverse transcription into cDNA, the fusion gene transcripts are used as polymerase chain reaction (PCR) targets for quantitative analysis of minimal residual disease (MRD). Primer and probe sets for real-time quantitative PCR (RQ-PCR) detection of the most common fusion gene transcripts as well as control gene transcripts have been designed (Figure 1) and reach sensitivities of at least 10^{-4}.[1,2]

Junctional regions of rearranged immunoglobulin (Ig) and T-cell receptor (TCR) genes represent unique 'fingerprint-like' sequences, which are assumed to be different in each lymphoid cell and lymphoid malignancy.[3] Therefore, junctional regions of Ig and TCR genes can be used as patient-specific targets for MRD-PCR analysis. After confirming clonality and sequencing of the rearrangement, junctional region-specific oligonucleotides can be designed. Germline primer and probes for RQ-PCR analysis have been designed for *IGH*, *IGK*-Kde, *TCRG*, *TCRD*, *TCRB*, and Vδ2-Jα rearrangements. These primer-probe sets in combination with junctional region-specific primers can generally reach sensitivities of 10^{-4}.[4]

RQ-PCR allows accurate quantification of PCR products during the exponential phase of the PCR amplification. During and after each subsequent PCR cycle, fluorescent signals are detected, and quantitative data can be obtained in a short period of time without the need of post-PCR processing. One principle of RQ-PCR detection is based on the use of hydrolysis probes which exploit the 5'→3' exonuclease activity of the *Thermus aquaticus* (*Taq*) polymerase to detect and quantify specific PCR products (Figure 2).[5] The hydrolysis probe (also referred as TaqMan probe) is conjugated with a reporter and a quencher fluorochrome, and should be positioned within the target sequence. As long as the probe is intact and both fluorochromes are in each other's close vicinity, the fluorescence emitted by the reporter fluorochrome will be 'absorbed' by the quencher fluorochrome. However, upon amplification of the target sequence, the hydrolysis probe is initially displaced from the DNA strand by the *Taq* polymerase and subsequently hydrolysed by the 5'→3' exonuclease activity of the *Taq* polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently, the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle the amount of fluorescent signal will further increase because of the exponential accumulation of free reporter fluorochromes. Based on the background fluorescence intensity, a cut-off level can be determined for specific fluorescence. This threshold is used to calculate the cycle threshold (C_T) of each sample (Figure 3A). To calculate the amount of template for each patient sample, a standard curve prepared from a dilution series from a diagnostic sample or a plasmid for both the target and control gene, can be used (Figure 3B). The target gene amount is subsequently divided by the control gene amount to obtain a normalized target value.

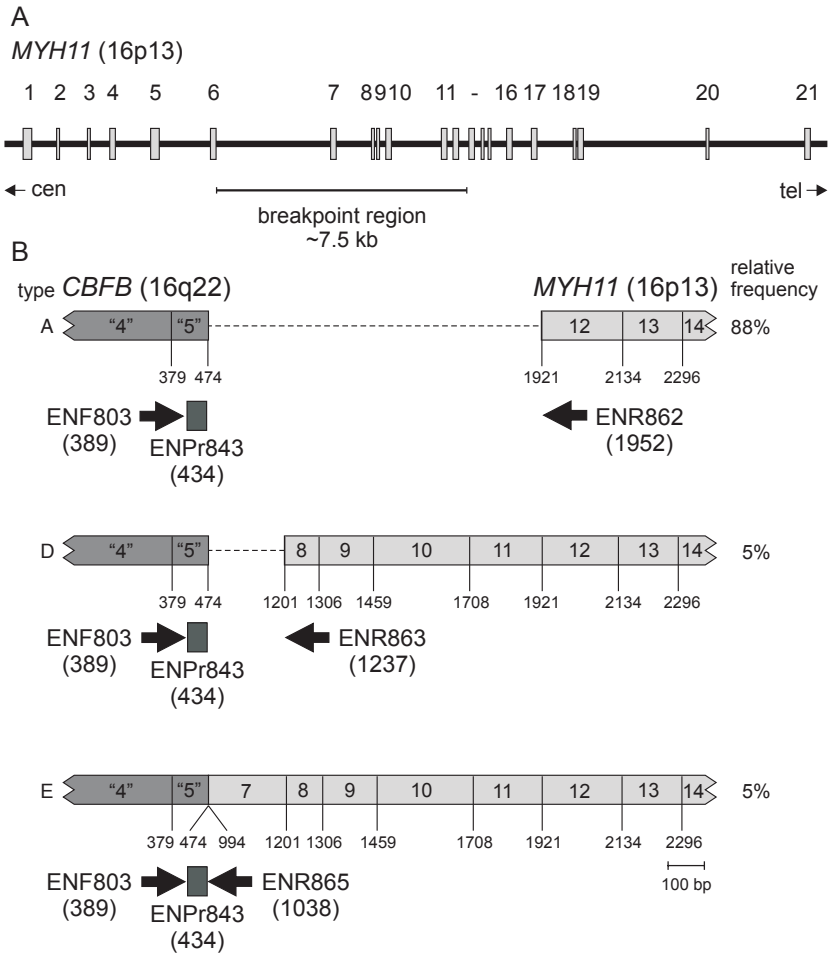


Figure 1.
(A) Schematic diagram of the exon/intron structure of the *MYH11* gene, which is involved in the inv(16)(p13q22). The centromere (cen) and telomere (tel) orientation, exon numbering, and the breakpoint region are indicated.
(B) Schematic diagram of the 3 most frequent types of *CBFB-MYH11* fusion transcripts (type A, D and E) covered by the RQ-PCR primers and probe set of the Europe Against Cancer program.[1] The relative frequency of each particular transcript appears on the right side of the diagram. The different types of transcripts are mainly caused by breakpoints in different introns of the *MYH11* gene. The numbers under the fusion gene transcript refer to the first (5') nucleotide of the involved gene, except when the last (3') nucleotide of the upstream gene is indicated. The number under the primers and probe refers to their 5' nucleotide position in the normal gene transcript. ENF = European network forward primer, ENPr = European network TaqMan probe, ENR = European network reverse primer.

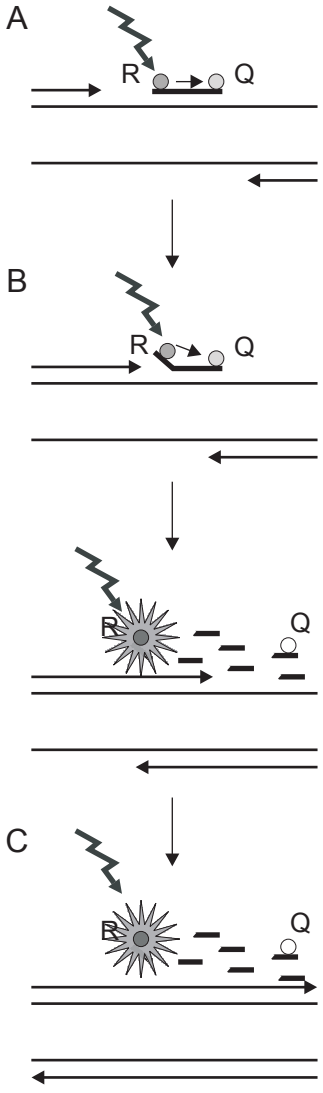


Figure 2. Principle of RQ-PCR detection using the hydrolysis probe technique.
(A) During the annealing phase, the double-dye oligonucleotide probe hybridizes to the complementary nucleotide sequence of the template during the PCR reaction.
(B) The probe is degraded during the extension phase by the DNA Taq polymerase enzyme, resulting in a fluorescent signal in the PCR tube detected by the machine.
(C) End of PCR cycle. R: reporter dye, Q: quencher dye.

In this part of the thesis, PCR-based MRD-targets in myeloid leukemia were studied. Previous studies using Southern blot analysis had shown the occurrence of cross-lineage Ig/TCR gene rearrangements in AML.[6] For RQ-PCR MRD studies, the precise sequence of the junctional region of the rearranged Ig/TCR genes needs to be known. Therefore, we performed a precise characterization of the type of Ig/TCR gene rearrangements that occur in AML (see Chapter 2.2). Secondly, we

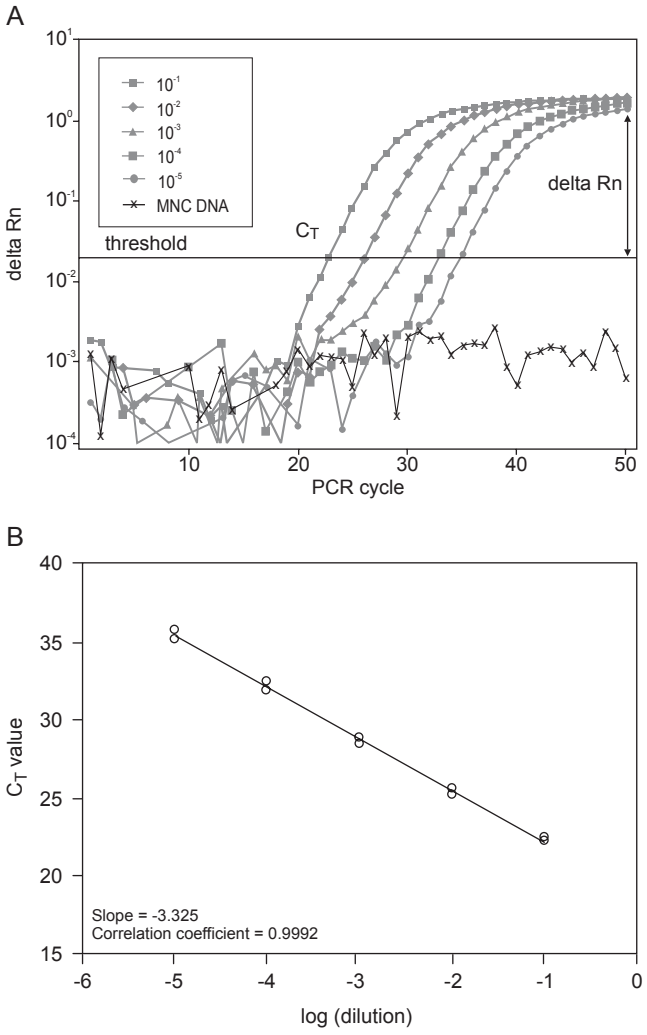


Figure 3.
(A) Amplification plot of several 10-fold dilutions of a diagnostic leukemia sample. The samples were diluted in normal mononuclear cell (MNC) RNA. Based on the background fluorescence intensity detected during the first three to 15 PCR cycles, a threshold is determined. The C_T is defined as the PCR cycle at which the fluorescent signal exceeds the threshold for the first time. The C_T value is directly proportional to the amount of target sequence present at the beginning of the reaction. The deltaRn corresponds to the increase in fluorescence intensity when the plateau phase is reached.
(B) Standard curve prepared from the data in (A). The slope of the standard curve is close to the theoretical slope of -3.32 for a PCR reaction with maximum efficiency. Unknown samples can be plotted in the standard curve. Based on their C_T value, the amount of template can be calculated.

investigated whether Ig/TCR gene rearrangements and chromosome aberrations with fusion gene transcripts are complementary targets for PCR-based MRD studies in AML (see Chapter 2.2). Additionally, we developed a RQ-PCR for the analysis of *RAG1* and *RAG2* gene expression levels as a potential tool for the selection of AML samples with Ig/TCR gene rearrangements (see Chapter 2.2).

Similar to rearranged Ig and TCR genes, fusion genes of chromosome aberrations at the DNA level are different in each patient, thereby representing patient-specific targets for PCR-based MRD monitoring. We focussed on the identification of genomic *BCR-ABL* e19a2 fusions (see Chapter 2.4).

Finally, as difference in stability over-time between fusion gene transcripts and control gene transcripts may result in an over- or underestimation of the MRD levels, we investigated the stability over-time of the fusion gene as well as the control gene transcripts (see Chapter 2.3).

REFERENCES

1. Gabert J, Beillard E, Van der Velden VHJ, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cave H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, Gonzalez M, Viehmann S, Malec M, Saglio G, Van Dongen JJM. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. *Leukemia* 2003; **17**: 2318-2357.
2. Beillard E, Pallisgaard N, Van der Velden VHJ, Bi W, Dee R, Van der Schoot E, Delabesse E, Macintyre E, Gottardi E, Saglio G, Watzinger F, Lion T, Van Dongen JJM, Hokland P, Gabert J. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. *Leukemia* 2003; **17**: 2474-2486.
3. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. *Clin Chim Acta* 1991; **198**: 1-91.
4. Van der Velden VHJ, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, Van Dongen JJM. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* 2003; **17**: 1013-1034.
5. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci USA* 1991; **88**: 7276-7280.
6. Adriaansen HJ, Soeting P, Wolvers-Tettero ILM, Van Dongen JJM. Immunoglobulin and T-cell receptor gene rearrangements in acute non-lymphocytic leukemias. Analysis of 54 cases and a review of the literature. Leukemia 1991; **5**: 744-751.

CHAPTER 2.2

Fusion gene transcripts and Ig/TCR gene rearrangements are complementary but infrequent targets for PCR-based detection of minimal residual disease in acute myeloid leukemia

Nancy Boeckx[1,2], Marja J. Willemse[1], Tomasz Szczepański[1,3], Vincent H.J. van der Velden[1], Anton W. Langerak[1], Philippe Vandekerckhove[2], Jacques J.M. van Dongen[1]

[1]Department of Immunology, Erasmus University Rotterdam/ University Hospital Rotterdam, Rotterdam, The Netherlands
[2]Department of Laboratory Medicine, Laboratory of Haematology, University Hospital Gasthuisberg, Leuven, Belgium
[3]Departement of Pediatric Haematology and Chemotherapy, Silesion Academy of Medicine, Zabrze, Poland

Published in Leukemia 2002; 16: 368-375.

ABSTRACT

PCR-based monitoring of minimal residual disease (MRD) in acute leukemias can be achieved via detection of fusion gene transcripts of chromosome aberrations or detection of immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements. We wished to assess whether both PCR targets are complementary in acute myeloid leukemia (AML).

We investigated 105 consecutive AML cases for the presence of fusion gene transcripts by reverse transcriptase polymerase chain reaction (RT-PCR): *AML1-ETO* associated with t(8;21), *CBFB-MYH11* with inv(16), *PML-RARA* with t(15;17), *BCR-ABL* with t(9;22), and *MLL-AF4* with t(4;11). In 17 out of 105 AML cases (16%), fusion gene transcripts were found. Ninety-five of these AML patients (13 with fusion gene transcripts) were also investigated for the presence of *IGH*, *IGK*, *TCR*, and *TCRD* rearrangements by Southern blot and/or PCR-heteroduplex analysis and sequencing. In nine out of 95 patients (9.5%) such rearrangements were found. Combined data revealed that only one patient with a fusion gene transcript had a coexistent Ig/TCR rearrangement. The nine AML patients with Ig/TCR rearrangements, as well as five additional AML patients from a previous study were investigated in more detail, revealing that Ig/TCR rearrangements in AML are immature and unusual. The presence of Ig/TCR rearrangements in AML did not correlate with *RAG* gene expression levels as determined by real-time quantitative PCR.

In conclusion, fusion gene transcripts and Ig/TCR rearrangements are infrequent, but complementary MRD-PCR targets in AML.

INTRODUCTION

Over the past decade, advances in therapeutic approaches have improved the outcome for patients with acute myeloid leukemia (AML). About 75% of patients under 65 years and about 50% of elderly patients achieve a complete remission, cytomorphologically defined as the presence of less than 5% blast cells in the bone marrow (BM). However, many patients relapse, implying that treatment does not kill all AML cells and that surviving cells remain undetected by the use of cytomorphology. To trace low frequencies of remaining leukemic cells, i.e. 'minimal residual disease' (MRD), more sensitive methods are required. MRD techniques should reach sensitivities of at least 10^{-3} (one leukemic cell in 1000 normal cells), but sensitivities of 10^{-4} to 10^{-6} are preferred.[1] Flow cytometric immunophenotyping and polymerase chain reaction (PCR) techniques are able to detect such low frequencies of leukemic cells. Detection of MRD by flow cytometry is based on the presence of aberrant phenotypic features of the AML cells that allow them to be distinguished from normal cells.[2] Monitoring of MRD by PCR techniques is

based on the detection of clone-specific sequences, such as junctional regions of immunoglobulin (Ig) and T cell receptor (TCR) genes or on the detection of leukemia-specific breakpoint fusion gene transcripts of chromosome aberrations.

Junctional regions of Ig and TCR gene rearrangements are the result of recombination of different variable (V), diversity (D), and joining (J) gene segments with deletions and random insertions of nucleotides at the coupling sites of the gene segments. This lymphoid-specific process is mediated via V(D)J recombinase activity, wherein the recombination activating gene products (RAG1 and RAG2) play a crucial role. Clonal patient-specific Ig and TCR gene rearrangements can be identified in the vast majority of pediatric and adult patients with acute lymphoblastic leukemia (ALL).[3,4] Cross-lineage Ig and TCR gene rearrangements have been reported in AML patients as well. Results from the literature reveal that Ig heavy chain (*IGH*) gene rearrangements occur in 4 to 18% of AML patients, whereas Ig kappa light chain (*IGK*) gene rearrangements occur in 1 to 5% only.[5-10] So far, only one AML case has been reported having rearrangements of the Ig lambda (*IGL*) locus.[10] Rearrangements of the TCR gamma (*TCRG*) and TCR delta (*TCRD*) genes have both been found in 4 to 14% of cases.[9-12]

Several types of PCR-detectable chromosome aberrations are relatively frequently found in AML: t(8;21)(q22;q22) occurs in 6-8% of adult AML and 10-14% of childhood AML and can especially be found in the AML-M2 subtype; inv(16)(p13;q22) occurs in 5-7% and is closely associated with the AML-M4Eo subtype; t(15;17)(q22;q21) is found in 5-10% of AML and is exclusively found in acute promyelocytic leukemia (AML-M3); about 5% of *de novo* AML have 11q23 abnormalities involving the *MLL* gene, such as t(4;11)(q21;q23) and t(9;11)(p22;q23).[13-15] However, t(9;22)(q34;q11) is rarely found in AML.[15] Chromosome aberrations can be used for risk group classification: 11q23 aberrations are associated with poor prognosis, whereas t(8;21), t(15;17) and inv(16) are associated with relatively good prognosis.[16,17]

Since previous studies mainly focused on the detection of either chromosome aberrations or Ig/TCR gene rearrangements, we now investigated the occurrence of both types of MRD-PCR targets in a large group of 105 consecutive AML patients. The aim of the here presented study was two-fold: (1) precise characterization of the type of Ig/TCR gene rearrangements that occur in AML patients; and (2) analysis of whether Ig/TCR gene rearrangements and chromosome aberrations with fusion gene transcripts are complementary PCR targets for MRD studies. Additionally, we developed a “real-time” quantitative polymerase chain reaction (RQ-PCR) using TaqMan technology for the analysis of *RAG1* and *RAG2* gene expression levels in AML patients, as a potential tool for selection of AML samples with Ig/TCR gene rearrangements.

MATERIALS AND METHODS

Patients and cell samples

Peripheral blood (PB) or BM samples from 105 consecutive AML patients (81 adults and 24 children) were obtained at initial diagnosis. The diagnosis of AML was based on cytomorphology, cytochemistry, and flow cytometric immunophenotyping. Myeloid blast crises of chronic myeloid leukemia were excluded from the study. Patients were classified according to the revised French-American-British (FAB) criteria.[18] Mononuclear cells (MNC) were isolated by Ficoll-Paque centrifugation (density: 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) and stored in liquid nitrogen.

Additional molecular analysis was performed on five previously described AML patients with Southern blot-detectable Ig/TCR gene rearrangements of which DNA was still available.[9] Three other patients from this previous study could not be further investigated due to lack of DNA.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared from cryopreserved MNC by phenol-chloroform extraction.[19] RT-PCR was performed on all 105 AML patients as previously described.[15] Samples were investigated for the presence of six fusion gene transcripts associated with well-defined chromosome aberrations: *AML1-ETO*, *CBFB-MYH11*, *PML-RARA*, *BCR-ABL* p190 and p210 and *MLL-AF4*. Quality of RNA was checked by amplification of *ABL* gene transcripts. A single PCR was performed in a volume of 50 µl using 2 µl of cDNA. The sequences of the primers used for PCR amplification were developed during the BIOMED-1 Concerted Action.[15] PCR conditions comprised: initial denaturation at 95°C for 30 s, followed by 35 cycles of 30 s at 94°C, 60 s at 65°C and 60 s at 72°C using a Perkin Elmer 9600 thermal cycler (Applied Biosystems, Foster City, CA, USA). Appropriate positive and negative controls were used in all experiments.[15]

Southern blot analysis of Ig/TCR gene rearrangements

Screening for *IGH*, *IGK*, and *TCRD* gene rearrangements was done by Southern blot analysis. DNA was isolated from frozen MNC fractions using the QIAamp Blood Kit (Qiagen, Chatsworth, CA, USA).[20] Fifteen *µ*g of DNA was digested with *Bgl*II (Gibco Ltd. Paisley, Scotland), size-separated in 0.7% agarose gels, and transferred by vacuum blot to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany), as described.[21] The membranes were hybridized with ^{32}P-random oligonucleotide-labeled probes. The *IGH* gene configuration was analyzed with the IGHJ6 probe (DAKO Corp, Carpinteria, CA, USA),[22] *IGK* gene rearrangements were analyzed with the IGKJ5 and IGKDE probes (DAKO),[23] and the *TCRD* gene configuration was analyzed with the TCRDJ1, TCRDJ3 and TCRDV2 probes (DAKO).[24]

Appropriate analysis of *TCRG* gene rearrangements can not be performed on *Bgl*II restricted DNA.[25] Because of limited availability of DNA, an additional digest with *Eco*RI (Gibco) was only performed in patients who showed clonal *TCRG* gene rearrangements by PCR heteroduplex analysis (see below). The *TCRG* gene configuration in these patients was analyzed with the TCRGJ13 probe (DAKO).[25]

PCR heteroduplex analysis of Ig/TCR gene rearrangements

Only patients who showed rearrangements and/or deletions of *IGH*, *IGK*, and/or *TCRD* genes on Southern blot were further investigated by PCR heteroduplex analysis for these genes. Southern blotting for detection of *TCRG* gene rearrangements was performed in a limited number of patients, but PCR heteroduplex analysis was performed in all patients. All relevant PCR analyses were also performed on the five AML samples from a previous Southern blot study, which showed Ig/TCR gene rearrangements.[9] PCR was essentially performed as described previously.[26] Briefly, in each 50 µl PCR reaction, 50 ng DNA sample, 6.25 pmol of the 5' and 3' oligonucleotide primers and 1 U AmpliTaq Gold polymerase (Applied Biosystems) were used. The sequences of the oligonucleotides used for amplification of *IGH*, *IGK*, *TCRG*, and *TCRD* gene rearrangements were described elsewhere.[26,27] PCR conditions comprised: initial 10 min at 94ºC, followed by 35 cycles of 45 s at 92ºC, 90 s at 60ºC and 2 min at 72ºC using a Perkin-Elmer 480 thermal cycler (Applied Biosystems). A final extension step of 10 min at 72ºC was performed after the last cycle. Appropriate positive and negative controls were used in all experiments.[26]

To confirm clonality of the PCR-amplified rearrangements, heteroduplex analysis was performed on positive PCR products.[28] PCR products were denaturated at 94ºC for 5 min and subsequent cooled at 4ºC for 60 min to induce duplex formation. The duplexes were immediately loaded on a 6% non-denaturating polyacrylamide gel in 0.5 x Tris-boric acid-EDTA (TBE) buffer, run at room temperature and visualized by ethidium bromide staining. A 100-bp DNA ladder (Promega Corp, Madison, WI, USA) was used as size marker.

Sequence analysis of Ig and TCR gene rearrangements

PCR products found to be clonal by heteroduplex analysis were directly sequenced, except for cases in which heteroduplex PCR analysis showed more than two clonal bands. In such cases, homo- or heteroduplex bands were excised from the polyacrylamide gel and eluted as described before.[27] The eluted PCR products were sequenced directly. Sequencing was performed using the BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase on an ABI PRISM 377 automated sequencer (Applied Biosystems). Briefly, 50 to 200 ng of PCR product, 3.2 pmol primer and 5 µl BigDye terminator mix were used in a 15 µl reaction volume. The cycling protocol was 96ºC for 30 s, followed by 60ºC for 4 min for a total of 25 cycles. All rearrangements were sequenced in two directions from two independent PCR products.

Interpretation of sequence data

V, D and J gene segments in *TCRG* and *TCRD* rearrangements were identified as described previously.[29] The Ig sequences were analyzed with the DNAPLOT software (Müller, H.H. Althaus, University of Cologne, Germany) by searching for homology with all known human germline sequences obtained from the VBASE directory of human Ig genes (http://www.mrc-cpe.cam.ac.uk/imt-doc/) and IMGT (http://imgt.cnusc.fr:8104/). Most sequences were additionally analyzed using BLAST sequence similarity searching tool (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/BLAST/).

Real-time quantitative PCR (RQ-PCR) of RAG gene expression levels

Based on germline RNA sequences of the human *RAG1* and *RAG2* genes, primers and TaqMan probes were designed using Primer-Express software (Applied Biosytems) according to the manufacturer's guidelines. *RAG1*: forward primer 5'-TGAGTAATATCAACCAAATTGCAGACA-3', reverse primer 5'-GGATCTCACCCGGAACAGC-3', TaqMan probe 5'-CCCCAGATGAAATTCAGCAC CCACATATTAA-3'; *RAG2*: forward primer 5'-GTTTAGCGGCAAAGATTCAGAGA-3', reverse primer 5'-GTCCATCAAAATTCATCAGTGAGAA-3', TaqMan probe 5'-CAAAAATCTACGTACCATCAGAAACTA TGTCTCTGCA-3' (Applied Biosystems).

Fifty µl reaction mixtures contained Universal Master Mix 2x (Applied Biosystems), 900 mM primers, 200 mM probe and approximately 100 ng of RNA (6 µl cDNA). RQ-PCR experiments were performed in duplicate and consisted of 2 min at 50ºC, 10 min at 95ºC, followed by 50 cycles of 15 s at 95ºC and 1 min at 60ºC. Real-time detection of obtained PCR products was based on TaqMan technology using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Non-template controls (NTC) and thymocyte DNA were used as negative controls in each *RAG1/RAG2* RQ-PCR experiment. In all cases, thymocyte DNA did not result in any amplification, indicating that *RAG1* and *RAG2* mRNA was specifically detected by our RQ-PCR method. A standard curve was established using a dilution series of thymocyte RNA (10^{-1} to 10^{-6}) in cell line RNA (HL-60). As HL-60 cells do not express *RAG1* or *RAG2*, HL-60 cDNA was taken as a control in each experiment and was always negative. To correct for differences in the amount of total RNA added to a reaction, the copy number was normalized to the *GAPDH* housekeeping gene (Applied Biosystems kit). The *GAPDH* assay was confirmed to be RNA specific, as thymocyte DNA included in each experiment was always negative. RQ-PCR data of *RAG1* and *RAG2* were subsequently corrected for the tumor load of the sample as determined by flow cytometric immunophenotyping.

Statistical analysis

A χ^2 or Fisher's exact test on a 2 x 2 table was performed for comparison of patient and disease characteristics (age, sex, FAB subtype and immunophenotype) between AML patients with Ig/TCR gene rearrangements *vs* patients without Ig/TCR gene rearrangements. A *P* value of <0.05 was regarded to be statistically significant.

A linear mixed model has been used to analyze differences in *RAG* gene expression as a function of the type of *RAG* gene (*RAG1* and *RAG2*), sample origin (BM or PB) and patient group (AML-germline, AML-rearranged and ALL). To model the correlation between the repeated measures per subject, a Kronecker product of two unstructured covariance matrices (one for sample origin, and one for the type of *RAG* gene) was used. The alpha-level 0.05 was used. All analyses were performed with the SAS-procedure PROC MIXED (version 8.1).

RESULTS

Detection of fusion gene transcripts in AML patients

Of 105 AML patients, 17 harboured chromosome aberrations (16%) (4/24 pediatric patients and 13/81 adults) as detected by RT-PCR analysis. Six patients (four children and two adults) had an *AML1-ETO* fusion gene transcript (5.7%): four concerned AML-M2, one AML-M4 and one AML-M1. *CBFB-MYH11* fusion gene transcripts were detected in six cases (5.7%); all six concerned the predominant type A fusion gene transcript.[15] Five of these six leukemias were classified as an AML-M4Eo and one as AML-M5. Three cases with *PML-RARA* fusion gene transcripts were found (2.9%): all these patients had an AML-M3 morphology. In two adult AML patients (1.9%), a *BCR-ABL* fusion gene transcript was identified: one encoded the 210 kDa fusion protein and one the smaller 190 kDa *BCR-ABL* protein. They corresponded with an AML-M0 and an AML-M4, respectively. An *MLL-AF4* fusion gene transcript was not found in this series. In the additional series of five AML patients from our previous study,[9] no fusion gene transcripts were detected by RT-PCR.

Detection of Ig/TCR rearrangements in AML

Ninety-five AML patients (74 adults and 21 children) were subjected to Southern blot analysis of the *IGH*, *IGK* and *TCRD* gene loci. Ten patients could not be studied because of insufficient amounts of DNA; coincidently, four of these 10 patients contained a fusion gene transcript. This is in contrast to only 13 of the 95 patients (14%), which were studied for Ig/TCR gene rearrangements.

IGH gene rearrangements were detected by Southern blotting in 5/95 AML patients (5%): two children and three adults (Figure 1). Monoallelic *IGH* gene rearrangements were found in three out of five patients (P-26, A-16, and A-34) and biallelic rearrangements were observed in case P-08. Southern blot analysis of patient A-62 showed three rearranged bands of different densities, suggesting the formation of subclones. Detailed PCR heteroduplex analysis of the *IGH* locus in the five patients with *IGH* gene rearrangements on Southern blot, detected seven clonal homoduplexes: one complete V_H-J_H rearrangement (patient P-08) and six

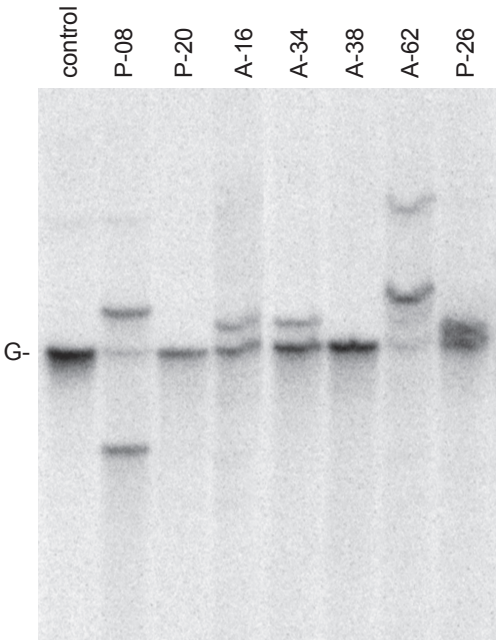


Figure 1. Southern blot analysis of the *IGH* gene in three pediatric and four adult AML patients. Control DNA and DNA from the seven AML patients were digested with *Bgl*II, size separated and blotted on to nylon membrane filters, which were hybridized with the ^{32}P labeled IGHJ6 probe. The *IGH* gene was rearranged in five out of the seven patients presented and a germline configuration was present in two patients (P-20 and A-38). Rearrangement of the *IGH* gene locus on one allele was found in three patients (A-16, A-34 and P-26) and on both alleles in one patient (P-08). In patient A-62, more than two rearranged *IGH* gene bands of different density were visible, suggesting the presence of subclones.

incomplete D_H-J_H rearrangements (Table 1). Sequence analysis revealed that the complete rearrangement concerned a V_H6-D_H1-J_H3b joining. In the incomplete rearrangements, the D_H3-22 gene segment was used preferentially (3/6 cases). The other three D_H segments used in the incomplete rearrangements were D_H1-26, D_H2-2 and D_H3-16. Except for one (D_H2-2), all identified D_H gene segments belong to the more downstream part of the D_H region. One Southern blot detectable *IGH* rearrangement (in P-08) could not be identified by PCR analysis with the primer set used (Table 1).

IGK gene rearrangements were found in only one patient (P-08): hybridization with the IGKJ5 probe showed a deletion, whereas hybridization with the IGKDE probe revealed a rearranged band. All other AML patients displayed the *IGK* locus in

Table 1. Junctional regions of the clonal Ig and TCR gene rearrangements in (A) 9/95 consecutive *de novo* AML patients with cross-lineage rearrangements and (B) five selected AML patients from a previous study.[9]

Gene locus	Patient code	Rearrangement	Junctional region[a]	
IGH[b]	P-08	V_H6-1-D_H1-J_H3b	0/TCT/-6/D_H1/-2/GGCGATTGC/-1	**A**
		Not identified		
	P-26	D_H3-16-J_H4b	-6/CTTGGATC/-4	
	A-16	D_H3-22-J_H4b	0/0/-8	
	A-34	D_H3-22-J_H4b	-10/CAC/-6	
	A-62	D_H1-26-J_H4b	-4/GGGTTG/-6	
		D_H2-2-J_H6c	-11/TCCC/-6	
		D_H3-22-J_H4b	-3/CCCC/-4	
IGK	P-08	Vk3-20-Kde[c]	0/GGGAGGGAGAT/0/Jk3-intron-RSSJk4/0/AGGCCGG/-3	
TCRD	P-08	Vδ2-Dδ3-Jδ1	-5/CCATT/0/Dδ3/0/CCCGATGT/0	
	P-20[d]	Dδ2-Dδ3	0/GGGGTG/-1	
	P-24	Biallelic deletion	not applicable	
TCRG[e]	P-08	Vγ9-Jγ2.3 (subclone)	-2/CACCAAAGGAGGGGGT/-3	
	A-25	Vγ10-Jγ2.3 (subclone)	-8/GATTAAGGGGGGG/-11	
		Vγ9-Jγ2.3 (subclone)	0/CCT/-14	
	A-38	Vγ9-Jγ2.3 (subclone)	0/CGGG/-12	
IGH[b]	RM	D_H2-21-J_H	NA[f]	**B**
	PM	D_H4-23 -J_H4b	0/GGTGGAAGG/-10	
		D_H7-27-J_H6b	-10/CGCGGGCT/-23	
	BM	D_H7-27-J_H5b	-2/0/-3	
	JC	Not identified		
TCRD	RS	Vδ2-Dδ3	-7/CCCGTCC/-1	
	PM	Dδ2-Jδ1	-1/AATACTGGGGGGCCCAGGGG/-3	
		Dδ2-Jδ1	-1/CCCGGTACTTAAAAATAGG/0	
TCRG	PM	Vγ8-J+γ2.3	-3/GGAAGG/-5	
		Vγ11-Jγ2.1	-7/GGG/-5	
	RS	Vγ11-Jγ2.3	-7/CTAGGGGA/-5	

[a] Given are the numbers of deleted nucleotides from the germline sequence of the involved gene segments and the sequence of the N-regions.

[b] The most recent nomenclature was used to designate *IGH* gene segments.[36]

[c] Complex *IGK* gene rearrangement: The Vk3-20 gene segment was coupled to Jk3, followed by the intronic sequence till the RSS of Jk4. The Jk4 gene segment itself was absent, the RSS of Jk4 was followed by another junctional region and Kde.

[d] This is the only AML patient who also had a fusion gene transcript.

[e] The four *TCRG* gene rearrangements were only detectable by PCR, not by Southern blotting, implying that they were derived from small subclones.

[f] NA, sequence information not available.

germline configuration. PCR heteroduplex analysis of patient P-08 showed a Vκ III-Kde rearrangement. The size of this PCR product appeared to be approximately 800 bp on agarose instead of the expected 429 bp (Figure 2). Sequence analysis revealed a complex rearrangement in which the Vκ3.20 gene segment was coupled to the Jκ3, followed by the intronic sequence until the RSS of Jκ4. The Jκ4 gene segment

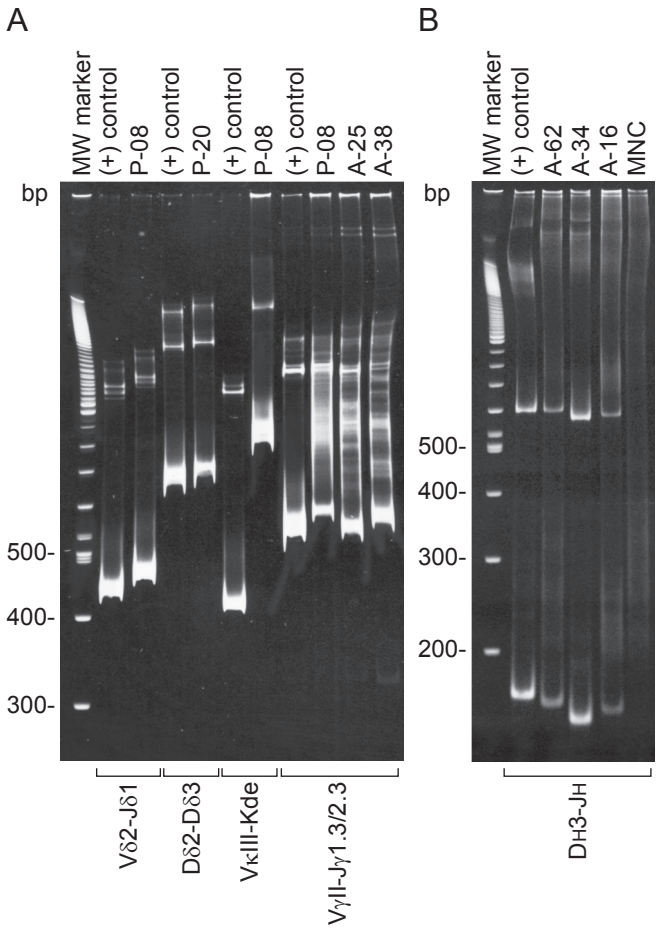


Figure 2. PCR heteroduplex analysis of cross-lineage rearrangements in AML.
Samples containing PCR products were subjected to heteroduplex PCR analysis, separated in a 6% polyacrylamide gel and visualized by ethidium bromide staining. **(A)** Clonal Vδ2-Jδ1 and Dδ2-Dδ3 homoduplexes were seen in patient P-08 and P-20, respectively. The VκIII-Kde combination showed a large clonal product in P-08. Analysis with VγII family-specific primers in combination with Jγ1.3/2.3 primers shows homoduplexes in three patients. **(B)** Using D_H3 primers in combination with J_H consensus primers, clonal PCR products were found in patients A-16, A-34 and A-62.

itself was absent, yet another junctional region, followed by Kde was found (Table 1). So, this seemingly Vκ III-Kde rearrangement is in fact a Jκ4-Kde rearrangement.

Analysis of the *TCRD* gene configuration in the 95 samples demonstrated rearrangements and/or deletions of this locus in three pediatric cases (P-08, P-20 and P-24). By PCR heteroduplex analysis, clonal *TCRD* PCR products were found in two patients: in patient P-08, the rearrangement consisted of a complete Vδ2-Dδ3-Jδ1 joining, whereas in patient P-20 an incomplete Dδ2-Dδ3 rearrangement was identified. In patient P-24 both *TCRD* alleles were deleted. *TCRG* gene rearrangements were generally not analyzed by Southern blotting, due to limited availability of DNA. Hence PCR heteroduplex analysis of *TCRG* was performed in all 95 AML patients (74 adults and 21 children). Four monoclonal rearrangements were found in three patients (A-25, A-38 and P-08). Subsequent direct sequence analysis of PCR products elucidated that in these four rearrangements the VγII (Vγ9) or VγIII (Vγ10) gene segment was joined to the Jγ2.3 gene segment. By Southern blotting, no rearranged bands of *TCRG* gene locus could however be detected in these three patients, indicating that all four clonal PCR products were probably derived from small subclones. The junctional regions of all identified Ig and TCR gene rearrangements showed deletions as well as insertions (Table 1).

Taking the Southern blot and PCR heteroduplex analysis data together, major cross-lineage *IGH*, *IGK* and/or *TCRD* gene rearrangements occurred in only 6/95 AML patients (Table 1). In only one patient (P-20) a fusion gene transcript (*AML1-ETO*) associated with a chromosome aberration was also found. Therefore, at least one reliable PCR target was available for MRD detection in 18 out of 95 AML patients (19%).

Identification of Ig/TCR gene rearrangements in an additional series of five AML patients

In our previous study,[9] 8/54 (15%) patients were shown to have Ig/TCR gene rearrangements by Southern blot analysis. Five of them were studied here by PCR heteroduplex analysis.

PCR-heteroduplex analysis of the *IGH* locus resulted in a total of four monoclonal D_H-J_H products in three out of four patients with Southern blot detected rearrangements. The D_H family gene segments used included D_H2, D_H4 and D_H7. Additional sequencing showed that the D_H gene segments belonged to the most downstream part of the D_H region. The rearranged allele detected by Southern blot analysis in patient JC, could not be identified with the used primer combinations (Table 1).

For the *TCRD* gene locus, a Vδ2-Dδ3 rearrangement was detected in patient RS by Southern blot analysis and confirmed by PCR heteroduplex analysis in this study. The other rearranged allele in this patient could not be identified. In patient PM, Southern blot analysis showed one rearranged allele. Using the Dδ2-Jδ1 primer combination, two heteroduplexes and co-migrating homoduplexes

were found by PCR heteroduplex analysis. Sequence analysis allowed us to identify both heteroduplexes (Table 1).

Biallelic rearrangements of the *TCRG* locus were found in two patients of the previous study.[9] PCR heteroduplex analysis allowed us to identify a VγI-Jγ2.3 and a VγIV-Jγ2.1 joining in patient PM, whereas only one of the two *TCRG* gene rearrangements found by Southern blot could be identified in patient RS (VγIV-Jγ2.3) (Table 1).

Association of Ig/TCR gene rearrangements with patient and disease characteristics

AML patients with Ig/TCR gene rearrangements and patients without Ig/TCR gene rearrangements were compared for their patient and disease characteristics (age, sex, FAB subtype and immunophenotype). Cytogenetic findings could not be included, since these data were incompletely. Results showed that two associations were statistically significant. Firstly, in the patient group of 16 to 60 years, the frequency of Ig/TCR gene rearrangements (2%) was significantly lower ($P = 0.041$) as compared to the other patient groups (<5 year, 5-15 years and >60 years). A second significant association was observed between the expression of B cell associated markers (CD19 and/or CD20) and the presence of Ig/TCR gene rearrangements ($P = 0.014$). All other immunophenotypic parameters, including expression of T cell-associated markers (CD2/CD4/CD5/CD7) and TdT, were not significantly related to the presence or absence of Ig/TCR gene rearrangements. Finally, no significant correlation was found between sex or FAB subtype and the occurrence Ig/TCR gene rearrangements.

Analysis of *RAG1* and *RAG2* expression levels

BM and/or PB of seven AML patients with Ig and/or TCR rearrangements could be analyzed for the level of *RAG1* and *RAG2* transcripts, in order to investigate whether the occurrence of Ig/TCR gene rearrangements in AML was related to *RAG* expression levels. Results were compared with the expression levels in nine AML patients without Ig/TCR gene rearrangements. ALL patients with monoclonal (n=10) or oligoclonal (n=10) rearrangements were used as positive control groups. Results showed that the *RAG1* expression was significantly lower than the *RAG2* expression in all four groups ($P < 0.05$). However, the *RAG1* and *RAG2* expression levels were not significantly different between the AML patients with rearranged Ig/TCR genes and the AML patients with germline genes. Furthermore, the *RAG1* and *RAG2* expression levels were significantly lower in AML with rearranged Ig/TCR genes than in ALL with monoclonal rearrangements and ALL with oligoclonal rearrangements ($P <0.0001$). *RAG1* and *RAG2* expression levels did not differ significantly between the two latter control groups. Finally, the expression levels of *RAG*1 and *RAG2* were not significantly different between BM and PB samples of the AML patients (Figure 3).

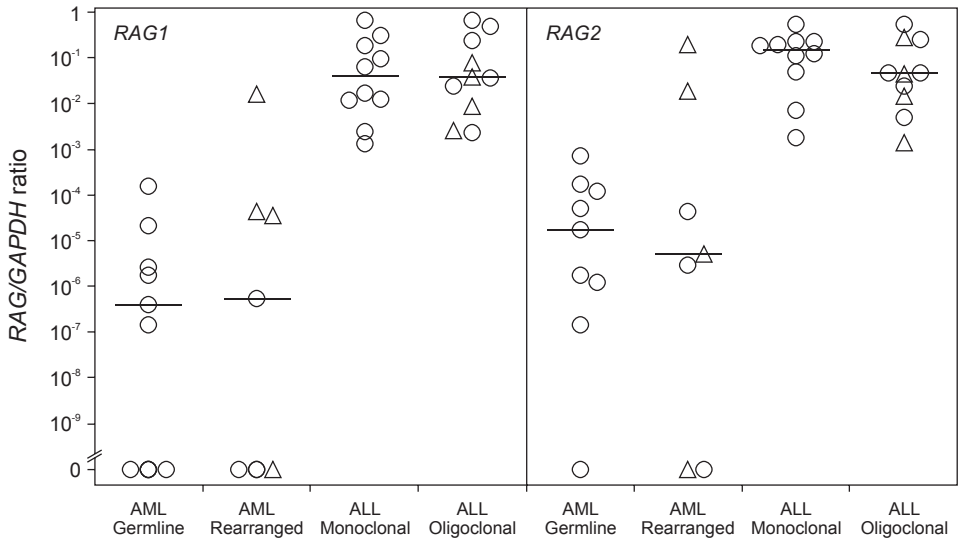


Figure 3. *RAG1* and *RAG2* gene mRNA levels in AML and ALL as assessed by RQ-PCR.
BM (circles) and/or PB (triangles) samples of AML with or without rearranged Ig/TCR genes were analyzed for their levels of *RAG1* and *RAG2* transcripts. Monoclonal and oligoclonal ALL were used as positive controls.

DISCUSSION

In our study, 105 consecutive AML patients were investigated by RT-PCR for the presence of fusion gene transcripts, which were identified in 17 AML patients (16%), including four of the 24 children (16.5%). This percentage in childhood AML is rather low compared with the frequencies published by other groups,[30] which may be caused by the relatively small number of children analyzed. In agreement with the findings of Raimondi *et al.*,[30] we found that t(8;21) is the most common aberration in pediatric patients. In the adult AML patients, we identified 13 fusion gene transcripts (16%). Although in adult AML the frequency of the investigated fusion gene transcripts is considered to be 20-25%, age-related differences can be observed. In AML patients above 60 years of age at diagnosis, the total frequency of detectable fusion genes is <10%.[31] In our study, 27 of the 81 adults belonged to this older age group and only one of them (4%) contained a fusion gene transcript (inv(16)) as detected by RT-PCR; this might partly explain the lower overall percentage in this study. Consequently, the frequency of fusion transcripts in the AML patients below 60 years was 22% (12/54). Two adult AML patients contained *BCR-ABL* transcripts, which is a remarkable finding because in most AML reports the frequency of t(9;22) is <1%.[15]

Cross-lineage Ig/TCR gene rearrangements were investigated in 95 out of the 105 AML patients. Southern blot analysis of the *IGH*, *IGK* and *TCRD* genes showed that clonal rearrangements were found in five, one and three cases, respectively, corresponding to seven different patients. These frequencies are somewhat lower than those found by other groups (Table 2).[9-12] In our previous study, we found *IGH*, *IGK* and *TCRD* gene rearrangements in 13%, 2%, and 4% of the patients, respectively. In six of the seven cases of our new patient series, we could identify the rearrangements with PCR heteroduplex analysis. Sequence analysis of these rearrangements showed that most junctional regions were comparable to what can be found in ALL. Hence, these Ig/TCR gene rearrangements are potentially suitable as PCR targets for MRD analysis.

Previous studies indicate that about 5% of AML cases have *TCRG* gene rearrangements as detected by Southern blot analysis.[9,10] In our series, 95 AML patients were prescreened by PCR heteroduplex for this target: four clonal rearrangements were identified in a total of three patients. However, no rearranged bands could be found by subsequent Southern blot analysis, probably because the clonal PCR products derived from a small leukemic subclone. We therefore assume that these *TCRG* gene rearrangements are not useful as PCR-MRD targets in AML.

To get more insight into the type of Ig/TCR gene rearrangements that occur in AML, we additionally performed PCR heteroduplex analysis in five AML patients, who were shown to contain *IGH* (n=4), *TCRD* (n=2), and/or *TCRG* (n=2) rearrangements by Southern blot analysis.[9] Looking at the *IGH* locus of both patient groups, 10 of the 11 gene rearrangements were incomplete D_H-J_H joinings (Table 1). Only one concerned a complete V_H-D_H-J_H recombination, involving the most downstream V_H6-1 gene segment. Virtually all used D_H gene segments (10/11; 91%) belonged to the more downstream D_H segments. Analysis of the J_H gene segment usage showed a more frequent usage of the J_H4 and J_H6 gene segments, which is also the case in normal lymphocytes and B cell malignancies. The predominance of incomplete D_H-J_H rearrangements together with the frequent usage

Table 2. Frequency of Ig and TCR gene rearrangements in AML.

References[a]	*IGH* genes	*IGK* genes	*TCRD* genes
Fontenay *et al*.[12]	10/57 (17%)	NT	8/57 (14%)
Adriaansen *et al*.[9]	7/54 (13%)	1/54 (2%)	2/54 (4%)
Sanchez *et al*.[10]	16/160 (10%)	8/160 (5%)	21/160 (13%)
Schmidt *et al*.[11]	NT	NT	9/100 (9%)
This study	5/95 (5%)	1/95 (1%)	3/95 (3%)
TOTAL	38/366 (10.4%)	10/309 (3.2%)	43/466 (9.3%)

[a] Only reports on large patient series (n>50) were used for this table.
NT Not tested.

of the more downstream D_H gene segments fits with immature rearrangements as also found in T-ALL.[27] The only *IGK* rearrangement identified comprised a complex *IGK* gene rearrangement, which is rare in B cell malignancies (AW Langerak, unpublished results), and has never described in normal cells before. This complex rearrangement can only be explained by a multistep process.[32] Irrespective of the subclonal origin of the four *TCRG* gene rearrangements in the random series of 95 AML patients, it is remarkable that the total of seven *TCRG* rearrangements all included one of the four most downstream Vγ gene segments. Finally, the frequency of *TCRD* gene rearrangements is in line with previous reports.[33,34] Two *TCRD* rearrangements concerned the immature incomplete Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements; in one patient biallelic Dδ2-Jδ1 rearrangements were detected and another patient contained a complete Vδ2-Dδ3-Jδ1 rearrangement. This is in contrast to precursor-B-ALL, where *TCRD* gene rearrangements generally do not concern Jδ1 segments. Thus, the *TCRD* gene rearrangements in AML are rather heterogeneous.

Overall results show that only in one patient (P-20), a PCR-detectable chromosome aberration coexisted with an Ig/TCR gene rearrangement. Therefore, cross-lineage Ig/TCR gene rearrangements in AML might be useful as supplementary MRD-PCR targets in addition to leukemia-specific chromosome aberrations.

As the frequency of PCR-detectable Ig/TCR rearrangements in AML appeared to be low (<10%), we aimed to analyze whether their presence can be predicted by relatively high expression levels of *RAG1* and *RAG2* transcripts, enzymes involved in the rearrangement processes. Our results obtained by RQ-PCR analysis showed that the expression levels of both *RAG1* and *RAG2* mRNA were comparable between AML patients with and without Ig/TCR gene rearrangements. Although these genes must have been expressed in an earlier phase of AML development, they might have been downregulated at time of diagnosis. Apparently, *RAG1* and *RAG2* mRNA levels are not a straightforward tool for the prediction of Ig/TCR gene rearrangements in AML (Figure 3).

We also checked for associations between Ig/TCR gene rearrangements and patient and disease characteristics, such as immunophenotype, especially B and T cell-associated markers. Our results showed that the frequency of Ig/TCR gene rearrangements (2%) in the patient group of 16 to 60 years was significantly lower as compared to other age groups. Another interesting finding was the significant association between positivity for B cell-associated markers (CD19 and/or CD20) and the presence of Ig/TCR gene rearrangements. Importantly, no significant association between TdT expression and the presence of Ig/TCR gene rearrangements was seen, although most Ig/TCR rearranged AML did have inserted nucleotides at their junctional region. Most probably, Ig/TCR gene rearrangements represent early post-oncogenic events, whereas the enzymatic activity of V(D)J recombinase (RAG1 and RAG2) and TdT is already downregulated at the time of diagnosis.

In conclusion, this study shows that fusion gene transcripts and Ig/TCR gene rearrangements are generally complementary, but relatively infrequent PCR targets for MRD detection in AML. Investigation of Ig/TCR gene rearrangements in AML patients is probably not realistic in a routine setting, because this provides only a limited number of additional MRD-PCR targets, and its presence cannot be predicted by *RAG* mRNA expression. Previous studies on Ig/TCR gene rearrangements used Southern blot analysis and might have given an overestimation of the number of MRD-PCR targets, since not all rearrangements found by Southern blotting can be identified by PCR.

In this study, only approximately 20% of the AML patients had a reliable MRD-PCR target (16% with chromosome aberrations and 6% with PCR-identified Ig/TCR gene rearrangements). Consequently, the detection of MRD in the majority of AML patients needs another reliable and sensitive technique. Multi-parameter flow cytometric immunophenotyping is the best suited alternative, since 70 to 80% of AML patients show an aberrant or infrequent immunophenotype at diagnosis.[1,35]

ACKNOWLEDGMENTS

We are grateful to Prof. Dr. R. Benner for his continuous support and to P.G. Hoogeveen, J.M. Wijkhuijs, D. Jacobs, E.J. van Gastel-Mol, and I.L.M. Wolvers-Tettero for their technical assistance and T.M. van Os for the preparation of the figures. A special thanks to S. Fieuws from the Biostatistical Centre (Leuven) for his support in the statistical analyses.

REFERENCES

1. Van Dongen JJM, Szczepanski T, De Bruijn MA, Van den Beemd MW, De Bruin-Versteeg S, Wijkhuijs JM, Tibbe GJ, Van Gastel-Mol EJ, Groeneveld K, Hooijkaas H. Detection of minimal residual disease in acute leukemia patients. *Cytokines Mol Ther* 1996; **2**: 121-133.
2. Macedo A, Orfao A, Vidriales MB, Lopez-Berges MC, Valverde B, Gonzalez M, Caballero MD, Ramos F, Martinez M, Fernandez-Calvo J, *et al*. Characterization of aberrant phenotypes in acute myeloblastic leukemia. *Ann Hematol* 1995; **70**: 189-194.
3. Szczepanski T, Langerak AW, Wolvers-Tettero ILM, Ossenkoppele GJ, Verhoef G, Stul M, Petersen EJ, De Bruijn MA, Van't Veer MB, Van Dongen JJM. Immunoglobulin and T cell receptor gene rearrangement patterns in acute lymphoblastic leukemia are less mature in adults than in children: implications for selection of PCR targets for detection of minimal residual disease. *Leukemia* 1998; **12**: 1081-1088.
4. Szczepanski T, Pongers-Willemse MJ, Langerak AW, Van Dongen JJM. Unusual immunoglobulin and T-cell receptor gene rearrangement patterns in acute lymphoblastic leukemias. *Curr Top Microbiol Immunol* 1999; **246**: 205-213.
5. Cheng GY, Minden MD, Toyonaga B, Mak TW, McCulloch EA. T cell receptor and immunoglobulin gene rearrangements in acute myeloblastic leukemia. *J Exp Med* 1986; **163**: 414-424.

6. Papadopoulos KP, Bagg A, Bezwoda WR, Mendelow BV. Clinical relevance of immunophenotypic and immunogenotypic analysis in acute non-lymphoblastic leukaemia. *S Afr Med J* 1989; **76**: 335-338.
7. Foa R, Casorati G, Giubellino MC, Basso G, Schiro R, Pizzolo G, Lauria F, Lefranc MP, Rabbitts TH, Migone N. Rearrangements of immunoglobulin and T cell receptor beta and gamma genes are associated with terminal deoxynucleotidyl transferase expression in acute myeloid leukemia. *J Exp Med* 1987; **165**: 879-890.
8. Boehm TL, Werle A, Drahovsky D. Immunoglobulin heavy chain and T-cell receptor gamma and beta chain gene rearrangements in acute myeloid leukemias. *Mol Biol Med* 1987; **4**: 51-62.
9. Adriaansen HJ, Soeting P, Wolvers-Tettero ILM, Van Dongen JJM. Immunoglobulin and T-cell receptor gene rearrangements in acute non-lymphocytic leukemias. Analysis of 54 cases and a review of the literature. *Leukemia* 1991; **5**: 744-751.
10. Sanchez I, San Miguel JF, Corral J, Martin C, Perez R, Gonzalez M, Canizo MC, Orfao A, Gonzalez-Sarmiento R. Gene rearrangement in acute non-lymphoblastic leukaemia: correlation with morphological and immunophenotypic characteristics of blast cells. *Br J Haematol* 1995; **89**: 104-109.
11. Schmidt CA, Oettle H, Neubauer A, Seeger K, Thiel E, Huhn D, Siegert W, Ludwig WD. Rearrangements of T-cell receptor delta, gamma and beta genes in acute myeloid leukemia coexpressing T-lymphoid features. *Leukemia* 1992; **6**: 1263-1267.
12. Fontenay M, Flandrin G, Baurman H, Loiseau P, Valensi F, Daniel MT, Sigaux F. T cell receptor delta gene rearrangements occur predominantly in immature myeloid leukemias exhibiting lineage promiscuity. *Leukemia* 1990; **4**: 100-105.
13. Martineau M, Berger R, Lillington DM, Moorman AV, Secker-Walker LM. The t(6;11)(q27;q23) translocation in acute leukemia: a laboratory and clinical study of 30 cases. EU Concerted Action 11q23 Workshop participants. *Leukemia* 1998; **12**: 788-791.
14. Harrison CJ, Cuneo A, Clark R, Johansson B, Lafage-Pochitaloff M, Mugneret F, Moorman AV, Secker-Walker LM. Ten novel 11q23 chromosomal partner sites. European 11q23 Workshop participants. *Leukemia* 1998; **12**: 811-822.
15. Van Dongen JJM, Macintyre E, Gabert JA, Delabesse E, Rossi V, Saglio G, Gottardi E, Rambaldi A, Dotti G, Griesinger F, Parreira A, Gameiro P, Diaz MG, Malec M, Langerak AW, San Miguel JF, Biondi A. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. *Leukemia* 1999; **13**: 1901-1928.
16. Mrozek K, Heinonen K, Bloomfield CD. Prognostic value of cytogenetic findings in adults with acute myeloid leukemia. *Int J Hematol* 2000; **72**: 261-271.
17. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, Rees J, Hann I, Stevens R, Burnett A, Goldstone A. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood* 1998; **92**: 2322-2333.
18. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposal for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol* 1976; **33**: 451-458.
19. Sambrook J, Fritisch E, Maniatis T. Molecular Cloning, a laboratory Manual: Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989.
20. Verhagen OJ, Wijkhuijs A, Van der Sluijs-Gelling AJ, Szczepanski T, Van der Linden-Schrever BE, Pongers-Willemse MJ, Van Wering ER, Van Dongen JJM, Van der Schoot CE. Suitable DNA isolation method for the detection of minimal residual disease by PCR techniques. *Leukemia* 1999; **13**: 1298-1299.
21. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. *Clin Chim Acta* 1991; **198**: 1-91.

22. Beishuizen A, Verhoeven M, Mol EJ, Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Detection of immunoglobulin heavy-chain gene rearrangements by Southern blot analysis: recommendations for optimal results. *Leukemia* 1993; **7**: 2045-2053.
23. Beishuizen A, Verhoeven M, Mol EJ, Van Dongen JJM. Detection of immunoglobulin kappa light-chain gene rearrangement patterns by Southern blot analysis. *Leukemia* 1994; **8**: 2228-2236.
24. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven MA, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies, and junctional diversity of T-cell receptor-delta gene rearrangements in acute lymphoblastic leukemia. *Blood* 1993; **82**: 3063-3074.
25. Moreau E, Langerak AW, Van Gastel-Mol EJ, Wolvers-Tettero ILM, Zhan M, Zhou Q, Koop BF, Van Dongen JJM. Easy detection of all T cell receptor gamma (TCRG) gene rearrangements by Southern blot analysis: recommendations for optimal results. *Leukemia* 1999; **13**: 1620-1626.
26. Pongers-Willemse MJ, Seriu T, Stolz F, d'Aniello E, Gameiro P, Pisa P, Gonzalez M, Bartram CR, Panzer-Grumayer ER, Biondi A, San Miguel JF, Van Dongen JJM. Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets: Report of the BIOMED-1 CONCERTED ACTION: Investigation of minimal residual disease in acute leukemia. *Leukemia* 1999; **13**: 110-118.
27. Szczepanski T, Pongers-Willemse M, Langerak AW, Harts WA, Wijkhuijs AJ, Van Wering ER, Van Dongen JJM. Ig heavy chain gene rearrangements in T-cell acute lymphoblastic leukemia exhibit predominant D_H6-19 and D_H7-27 gene usage, can result in complete V-D-J rearrangements, and are rare in T-cell receptor alpha beta lineage. *Blood* 1999; **93**: 4079-4085.
28. Langerak AW, Szczepanski T, Van der Burg M, Wolvers-Tettero ILM, Van Dongen JJM. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. *Leukemia* 1997; **11**: 2192-2199.
29. Breit TM, Van Dongen JJM. Unravelling human T-cell receptor junctional region sequences. *Thymus* 1994; **22**: 177-199.
30. Raimondi SC, Chang M, Ravindranath Y, Behm FG, Gresik MV, Steuber CP, Weinstein HJ, Carroll AJ. Chromosomal abnormalities in 478 children with acute myeloid leukemia: clinical characteristics and treatment outcome in a cooperative pediatric oncology group study-POG 8821. *Blood* 1999; **94**: 3707-3716.
31. Van der Velden VHJ, Schoch C, Langerak AW, Schnittger S, Hoogeveen PG, Van Dongen JJM. Low frequency of RT-PCR-detectable chromosome aberrations in relapsed acute myeloid leukemia (AML): implications for detection of minimal residual disease (MRD). *Br J Haematol* 2001; **113**: 1082-1083.
32. Seriu T, Hansen-Hagge TE, Stark Y, Bartram CR. Immunoglobulin kappa gene rearrangements between the kappa deleting element and Jkappa recombination signal sequences in acute lymphoblastic leukemia and normal hematopoiesis. *Leukemia* 2000; **14**: 671-674.
33. Schmidt CA, Przybylski G, Oettle H, Seeger KH, Ludwig WD, Siegert W. Heterogeneity of V delta 2 TCR delta gene rearrangements in acute myeloid leukemia. *Leuk Lymphoma* 1994; **16**: 73-77.
34. Schmidt CA, Przybylski G, Seeger K, Siegert W. TCR delta gene rearrangements in acute myeloid leukemia with T-lymphoid antigen expression. *Leuk Lymphoma* 1995; **20**: 45-49.
35. San Miguel JF, Ciudad J, Vidriales MB, Orfao A, Lucio P, Porwit-MacDonald A, Gaipa G, Van Wering E, Van Dongen JJM. Immunophenotypical detection of minimal residual disease in acute leukemia. *Crit Rev Oncol Hematol* 1999; **32**: 175-185.
36. Corbett SJ, Tomlinson IM, Sonnhammer ELL, Buck D, Winter G. Sequence of the human immunoglobulin diversity (D) segment locus: a systematic analysis provides no evidence for the use of DIR segments, inverted D segments, "minor" D segments or D-D recombination. *J Mol Biol* 1997; **270**: 587-597.

CHAPTER 2.3

Differential stability of control gene and fusion gene transcripts over time may hamper accurate quantification of minimal residual disease – a study within the Europe Against Cancer Program

Vincent H.J. van der Velden[1], Nancy Boeckx[2], Marcos Gonzales[3], Maria Malec[4], Gisela Barbany[5], Thomas Lion[6], Enrico Gottardi[7], Niels Pallisgaard[8], Emmanuel Beillard[9], Wim C.J. Hop[10], Patricia G. Hoogeveen[1], Jean Gabert[9], and Jacques J.M. van Dongen[1] on behalf of the Europe Against Cancer Program

[1] Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
[2] Department of Laboratory Medicine, Laboratory of Hematology, University Hospital Gasthuisberg, Leuven, Belgium
[3] Department of Hematology, Hospital Clínico Universitario,Salamanca, Spain
[4] Department of Pathology, Karolinska, Stockholm, Sweden
[5] Department of Medical Sciences, Present affiliation: Department of Genetics and Pathology, Uppsala University Hospital, Uppsala, Sweden
[6] Children's Cancer Research Institute, St. Anna Children's Hospital, Vienna, Austria
[7] Clinical and Biological Science, University of Turin, Ospedale San Luigi Gonzaga, Orbassano-Torino, Italy
[8] Department of Immuno-hematology, Aarhus University Hospital, Aarhus, Denmark. Present address: Institute of Pathology, Odense University Hospital, Odense, Denmark
[9] Department of Hematology Biology, Institut Paoli Calmettes. Present affiliation: Department of Biochemistry & Molecular Biology, Hôpital Universitaire Nord - IFR Jean Roche - Université de la Méditerranée, Marseille, France
[10] Department of Epidemiology and Biostatistics, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Adapted from letter. Leukemia 2004; 18: 884-886.

ABSTRACT

Quantification of minimal residual disease (MRD) using fusion gene transcripts provides prognostic information. As shipment of freshly collected samples to the PCR laboratory may take considerable time, the assessment of the over time transcript stability is crucial for reliable MRD studies. Differences in stability over time of fusion gene transcripts and control gene transcripts may result in an over- or underestimation of the MRD levels. We evaluated in diagnostic samples and MRD settings, the stability over time of the control gene transcripts in normal and leukemic cells, peripheral blood (PB) and bone marrow (BM) samples, and different types of leukemia, as well as the stability of fusion gene transcripts over time. Results indicated that transcripts are rapidly degraded *ex vivo* and that the degradation can differ between transcript type, between PB and BM, and between patients. Our results indicate the need for processing the samples on the day of sampling, or better, to collect the samples in tubes with an RNA stabilisator.

INTRODUCTION

During the last decade, several studies have shown that quantification of minimal residual disease (MRD) provides prognostic information that can be used for treatment decisions in individual patients. In acute lymphoblastic leukemia (ALL), MRD information obtained at early time points during therapy allows the recognition of high risk and low risk patients, who may benefit from treatment intensification or treatment reduction, respectively. In acute promyelocytic leukemia (APL) and chronic myeloid leukemia (CML), MRD information at specific time-points enables effective early treatment intervention.[1]

Quantitative MRD data with sensitivities of at least 10^{-4} can be obtained by real-time quantitative PCR (RQ-PCR) analysis using fusion gene transcripts associated with chromosomal abnormalities as MRD-PCR targets.[2] For this purpose, primer and probe sets have recently been developed within the Europe Against Cancer (EAC) program.[3] In addition, three control gene transcripts (abelson (*ABL*), b2-microglobine (*B2M*) and glucuronidase (*GUS*)) were selected based on their suitability for MRD studies in leukemic patients.[4]

Shipment of freshly collected patient samples to the MRD-PCR laboratories may take considerable time. Especially in multi-center studies it may take 1-3 days before the patient sample can be processed. During this period, the quality of the RNA present in the sample may deteriorate;[5] this can be due to induction of cell death with subsequent degradation of RNA, or to cellular changes resulting in altered stability or altered expression of transcripts. So far, only few studies have addressed the stability of fusion gene transcripts and control gene transcripts in patient samples.[6-7]

Differential stability over time of fusion gene transcripts and control gene transcripts may result in over- or underestimation of MRD levels. Therefore, careful assessment of the transcript stability is crucial for reliable MRD studies. We determined whether the stability of the control gene transcripts differed (1) between normal and leukemic cells, (2) between bone marrow (BM) and peripheral blood (PB) samples, and (3) between different leukemia (ALL, AML, and CML). Furthermore, we investigated the stability of fusion gene transcripts over time and evaluated whether this stability was comparable to the stability of control gene transcripts.

MATERIALS AND METHODS

Three different sample protocols were used in this study: *protocol 1* (whole BM and/or PB obtained at diagnosis or relapse; n=29), *protocol 2* (mononuclear cells (MNC) obtained at diagnosis or relapse, diluted (20x) in MNC of a healthy control to mimic an MRD setting; n=9), and *protocol 3* (whole BM and/or PB samples obtained during follow-up; n=26). In all three protocols, the cell sample was divided into five identical tubes. Tube 0 was processed immediately, whereas tube 1, 2, 3, and 4 were kept at room temperature and processed at day 1, 2, 3, and 4 after sample collection, respectively. On the day of processing, MNC were separated by Ficoll density centrifugation (protocol 1 and 3 only) and RNA was isolated from 2 x 10^6 MNC. cDNA and RQ-PCR reactions were performed according to the EAC protocol.[3]

Because the control gene transcripts were the topic of our study, their absolute copy numbers could not be used to correct for the quantity and quality of the RNA. Therefore, ratios between copy numbers of the different control genes were used to determine whether they showed comparable degradation rates. Statistical analysis (mixed model with type of sample protocol (1/2/3), sample type (BM/PB), leukemia type (ALL/AML/CML), and time (0/1/2/3/4 days) as variables) showed that for none of the three ratios (*ABL/B2M*, *ABL/GUS*, and *GUS/B2M*) the type of protocol had a significant effect, indicating that the stability of the control gene transcripts is comparable between leukemic cells and normal cells, also during therapy. Therefore, for subsequent analyses, data from the three different sample protocols were combined.

We subsequently analyzed the stability of fusion gene transcripts. It should be noted that at arrival of the sample in the laboratory it was not known whether the newly diagnosed leukemia harbored a fusion gene transcript (sample protocol 1 and 2) or whether MRD was present during follow-up (sample protocol 3). Consequently, the number of patients that could be analyzed for individual fusion gene transcript was generally low (one *MLL-AF4*; two *TEL-AML1*; three *BCR-ABL* p190; three *AML1-ETO*; two *PML-RARA*; one *CBFB-MYH11*; 18 *BCR-ABL* p210). Therefore, no statistical analysis was performed per type of fusion gene transcript and data were analyzed independent of the type of sample protocol and the sample type (except for *BCR-ABL* p210). As the fusion gene/control gene transcript ratio in follow-up samples or diluted diagnostic samples is obviously lower than the ratio in diagnostic samples, the ratio at day 0 was set at 1 and the ratio at the subsequent days was expressed relative to day 0.

In order to obtain data on the absolute degradation rate of gene transcripts over time, we evaluated the stability of the three control gene transcripts (*ABL*, *B2M*, and *GUS*) in PB samples obtained from healthy subjects, using an *in vitro* transcribed non-human RNA as an exogenous internal positive control in order to correct for variations in MNC number, RNA isolation, and cDNA synthesis. Whole PB samples (n=12) were divided into four tubes. From tube 1, MNC were immediately separated by Ficoll density centrifugation, counted, and lyzed (first step RNA isolation). Per 1 x 10^6 cells, 5 μl of exogenous control RNA (1000-fold diluted; kindly provided by Wanli Bi, Applied Biosystems, Foster City, CA, USA) were added, followed by completion of the RNA isolation, cDNA synthesis, and RQ-PCR analysis. Tubes 2-4 were processed identically at day 1-3.

RESULTS

Comparison of control gene transcript ratios between BM and PB samples showed significant differences (Figure 1). The *ABL/B2M* and *GUS/B2M* ratios differed significantly between PB and BM at day 1, day 2, and day 4, with higher ratios in BM (Figure 1A and B). In contrast, the *ABL/GUS* ratio did not differ between BM and PB (data not shown) and did not change significantly over time (Figure 1C). These effects were comparable between the three main leukemia types. Overall, these data indicate that (1) the stability of *ABL*, *GUS*, and *B2M* is comparable among ALL, AML, and CML cells; (2) *ABL* and *GUS* transcripts have a comparable degradation rate between BM and PB; and (3) *B2M* transcripts show a higher degradation rate than *ABL* and *GUS* transcripts in BM, but not in PB.

Although the mean stability of most fusion gene transcripts seemed to be comparable to the control genes (as reflected by a constant ratio around 1), large differences in stability could be observed between patients expressing the same fusion gene transcript. For example, the maximum and minimum fusion gene/control gene transcript ratio (i.e. the assumed MRD level) in individual samples could differ up to 25-fold for *AML1-ETO* (at day 2), more than 100-fold for *BCR-ABL* p210 normalized to *B2M* (day 2, 3, and 4), and up to 40-fold for *BCR-ABL* p210 normalized to *ABL* or *GUS* (day 2, 3, and 4). An example is shown in Figure 2 for the ratio between *BCR-ABL* p210 and *ABL* in PB samples of CML patients processed at five subsequent days. Apparently, the stability of the fusion gene transcripts can vary substantially between different patients. Consequently, MRD data obtained from samples that are not processed immediately after sampling may not accurately reflect actual fusion gene transcript levels.

Experiments to obtain data on the absolute degradation rate of gene transcripts over time, showed that the exogenous control normalized C_T values of all three control gene transcripts increased with time and generally an increase of 1 C_T was observed on consecutive days (data not shown). These results indicate that the absolute degradation rate is approximately 50% per day (i.e. an increase of the C_T value with one cycle) in PB MNC.

DISCUSSION

Although our data are based on a limited and heterogeneous set of samples, they clearly indicate that transcripts are rapidly degraded *ex vivo* and that the rate of degradation can differ between different types of transcripts, between PB and BM, and between patients. As such differential degradation will result in an over- or underestimation of MRD levels, samples should preferably be processed on the day of sampling; this processing should include at least the Ficoll density centrifugation-based separation of MNC and the cell lysis step of the RNA extraction. Even better,

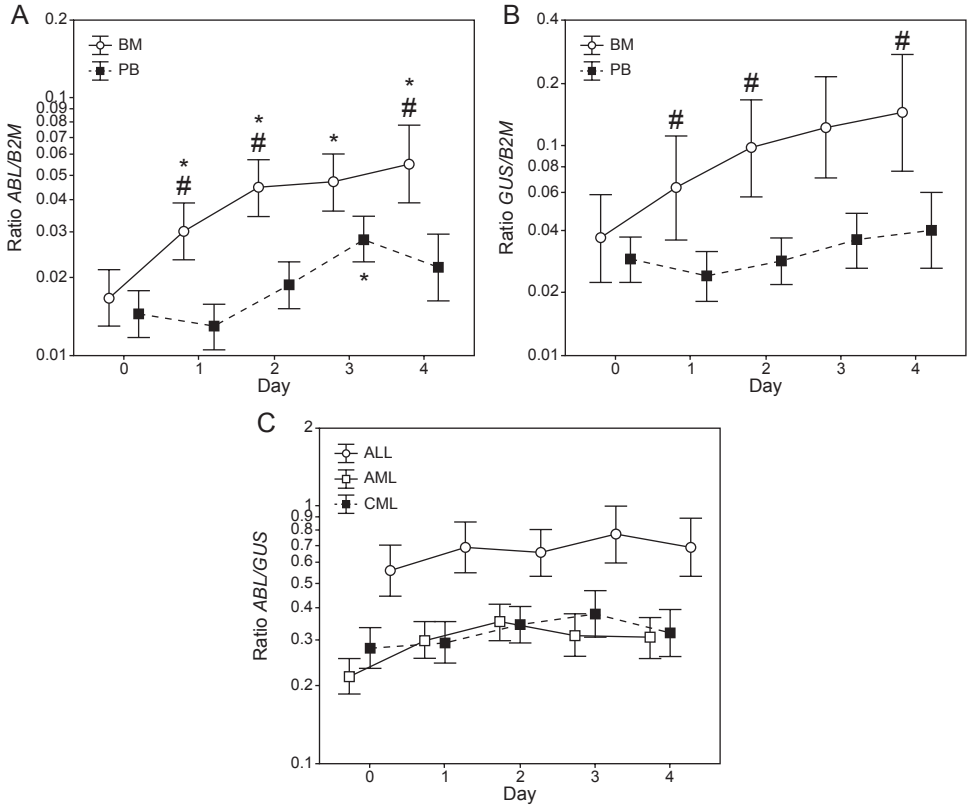


Figure 1. Stability of control genes over time.
Whole BM or PB samples were obtained from patients with leukemia at diagnosis or during follow-up. A total of 64 samples was obtained from 26 AML patients (21 BM, five PB), 22 CML patients (five BM, 17 PB), and 16 ALL patients (six BM, 10 PB; 12 precursor-B-ALL and four T-ALL patients).
(**A**) The *ABL/B2M* ratio differed significantly between BM and PB samples at day 1, 2, and 4. The *ABL/B2M* ratio in BM samples at day 1, 2, 3, and 4 was also significantly higher than at day 0. These effects were comparable between the three types of leukemia and therefore the combined data of the three leukemia types are shown.
(**B**) The *GUS/B2M* ratio in all three leukemia types was significantly different between BM and PB at day 1, 2, and 4. Because the *GUS/B2M* ratio was significantly lower in ALL than in AML and CML,[4] only the *GUS/B2M* ratio in ALL is shown.
(**C**) In agreement with previous results,[4] the *ABL/GUS* ratio was significantly higher in ALL as compared to AML and CML. Nevertheless, the *ABL/GUS* ratio did not differ between BM and PB and remained stable during the four day time period in all three leukemia types; consequently combined data of BM and PB samples are shown for each leukemia type. Symbols: *, P<0.05 relative to t=day 0; #, P<0.05 BM vs PB (mixed model analysis).

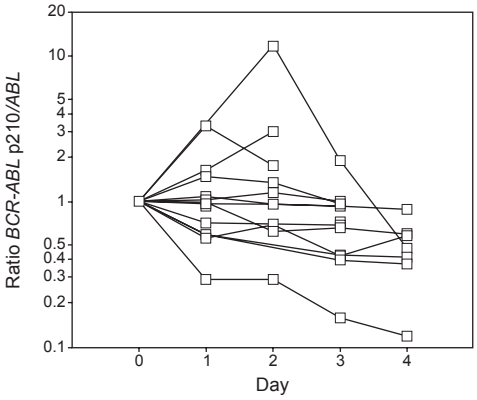


Figure 2.
Stability of *BCR-ABL* p210 fusion gene transcripts relative to *ABL* control gene transcripts in PB of CML patients (n=13). Although the mean *BCR-ABL/ABL* ratio remained relatively constant, individual cases showed large variations, with decreased ratios (up to 8.3 fold) in some cases but increased ratios (up to 11.8 fold) in others. This variation was not related with the type of sample protocol used (data not shown).

because changes in transcript levels can already occur within the initial 4 h,[5] samples should be collected in tubes with immediate stabilization of intracellular RNA, thereby preventing any degradation of control gene and/or fusion gene transcripts. Such reagents for stabilization of RNA have recently successfully been applied in a multi-center study.[8]

REFERENCES

1. Szczepanski T, Orfao A, Van der Velden VHJ, San Miguel JF, Van Dongen JJM. Minimal residual disease in leukaemia patients. *Lancet Oncol* 2001; **2**: 409-417.
2. Van der Velden VHJ, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, Van Dongen JJM. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* 2003; **17**: 1013-1034.
3. Gabert J, Beillard E, Van der Velden VHJ, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cavé H, Pane F, Aerts JLE, De Micheli D, Thirion X, Pradel V, González M, Viehmann S, Malec M, Saglio G, Van Dongen JJM. Standardization and quality control studies of “real-time” quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) of fusion gene transcripts or minimal residual disease detection in leukemia - A Europe Against Cancer Program. *Leukemia* 2003; **17**: 2318-2357.

4. Beillard E, Pallisgaard N, Van der Velden VHJ, Bi W, Dee R, Van der Schoot CE, Delabesse E, Macintyre E, Gottardi E, Saglio G, Watzinger F, Lion T, Van Dongen JJM, Hokland P, Gabert J. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using "real-time" quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - A Europe Against Cancer Program. *Leukemia* 2003; **17**: 2474-2486.
5. Tanner MA, Berk LS, Felten DL, Blidy AD, Bit SL, Ruff DW. Substantial changes in gene expression level due to the storage temperature and storage duration of human whole blood. *Clin Lab Haematol* 2002; **24**: 337-341.
6. Pallisgaard N, Clausen N, Schroder H, Hokland P. Rapid and sensitive minimal residual disease detection in acute leukemia by quantitative real-time RT-PCR exemplified by t(12;21) *TEL-AML1* fusion transcript. *Genes Chromosomes Cancer* 1999; **26**: 355-365.
7. Tobal K, Newton J, Macheta M, Chang J, Morgenstern G, Evans PA, Morgan G, Lucas GS, Liu Yin JA. Molecular quantitation of minimal residual disease in acute myeloid leukemia with t(8;21) can identify patients in durable remission and predict clinical relapse. *Blood* 2000; **95**: 815-819.
8. Mueller MC, Saglio G, Lin F, Pfeifer H, Press RD, Tubbs RR, Paschka P, Gottardi E, O'Brien SG, Ottmann OG, Hensley ML, Stockinger H, Ben-Am M, Wieczorek L, Hehlmann R, Hochhaus A. An international study to standardize the detection of *BCR-ABL* transcripts from stabilized peripheral blood preparations by quantitative RT-PCR. *Blood* 2003; **102**: 64a (abstract).

CHAPTER 2.4

Identification of e19a2 *BCR-ABL* fusions (*μ*-BCR breakpoints) at the DNA level by ligation-mediated PCR

Nancy Boeckx[1], Mieke W.J.C. Jansen[2], Cedric Haskovec[3], Peter Vandenberghe[4], Vincent H.J. van der Velden[2], Jacques J.M. van Dongen[2]

[1]Department of Laboratory Medicine, Laboratory of Hematology, Univerity Hospital Gasthuisberg, Leuven, Belgium
[2]Department of Immunology, Erasmus MC, Rotterdam, The Netherlands
[3]Institute of Hematology and Blood Transfusion, Prague, Czech Republic
[4]Department of Human Genetics, KU Leuven, Leuven, Belgium

Adapted from letter. Leukemia 2005; 19: 1292-1295.

ABSTRACT

BCR-ABL rearrangements represent the hallmark of chronic myeloid leukemia (CML). The breakpoints are usually clustered in the major breakpoint cluster region (BCR) and only sporadically, cases with breakpoints in the micro-BCR (*μ*-BCR) region are reported. In a new case of CML showing a *μ-BCR-ABL* rearrangement, we identified the DNA breakpoint sequence of the e19a2 junction using a ligation-mediated polymerase chain reaction (LM-PCR). Two other e19a2 junctions (one in a previously reported AML patient and one in the AR230 cell line) were also identified using this LM-PCR approach.

Breakpoints within the *μ*-BCR were located within a 393 bp stretch in the downstream part of intron 19, while *ABL* breakpoints were scattered over a ~24 kb region. At the coupling of the *BCR* and *ABL* intronic sequences, no insertions or duplications of sequences were found. Notably, in two out of three cases the break in the *ABL* gene was located within or near an *Alu* element.

This is the first report in which the exact DNA breakpoint sequences in e19a2 junctions are identified by LM-PCR. Such DNA sequences may potentially be used as patient-specific PCR targets for monitoring minimal residual disease.

INTRODUCTION

The Philadelphia chromosome (Ph) derives from a balanced translocation between chromosomes 9 and 22, and results in a *BCR-ABL* fusion gene. In the vast majority of Ph+ leukemias, breakpoints in the *ABL* gene are distributed over a ~200 kb breakpoint region between exons 1b and a2, while breakpoints in the *BCR* gene are clustered in three well-defined regions.[1] The major breakpoint cluster region (M-BCR) is a ~2.9 kb chromosomal region between exons 13 and 15.[1] The breakpoint can be detected in more than 95% of chronic myeloid leukemia (CML) and can lead to two types of mRNA molecules (e13a2 or e14a2, formerly assigned as b2a2 and b3a2), both encoding a $p210^{BCR-ABL}$ fusion protein. The minor breakpoint cluster region (m-BCR) spans a ~55 kb intronic sequence between the two alternative exons 1 and 2. The corresponding e1a2 fusion gene transcript encodes the $p190^{BCR-ABL}$ fusion protein and is frequently associated with Ph+ acute lymphoblastic leukemia. The micro-breakpoint cluster region (*μ*-BCR) spans a ~1 kb sequence which is located in intron 19 of the *BCR* gene. The corresponding fusion gene transcript e19a2 results in a 230 kDa protein. The e19a2 fusion gene was initially described to be associated with neutrophilic-CML (N-CML), a mild Ph+ myeloproliferative disease with only rarely progression, but in later reports e19a2 fusion gene transcripts were also found in classical CML and in acute myeloid leukemia (AML).[2,3]

Here, we report in a new case of CML with an e19a2 transcript the fusion at

the DNA level between *BCR* and *ABL* genes by using ligation-mediated PCR (LM-PCR). Additionally, the genomic *µ-BCR-ABL* breakpoints in an e19a2-positive AML and the AR230 cell line were identified.

PATIENTS, MATERIALS AND METHODS

Clinical course

In January 1999, a 69-year-old female was referred to the hospital with a leucocytosis of 130 x 10^9/l and immature granulocyte precursors. A bone marrow (BM) aspirate showed an increased cellularity with a myeloid/erythroid ratio of 10. Cytogenetic analysis revealed three pathological clones: 46,XX,t(9;22)(9q34;22q11)[10]/47,XX,idem,+der(22)t(9;22)[2]/46,XX,der(9)t(9;22),idic(22)(22pter→22q11:9q34:9q34:22q11→22pter)[2], supporting the diagnosis of CML in acceleration. An e19a2 fusion gene was identified by RT-PCR analysis using a BCR exon 19 primer (Table 1) and two previously described ABL exon a3 primers.[1] She was treated with hydroxyurea (HU) and showed a hematologic response. In May 2002, her BM showed 11% blasts. Gleevec® was started, but soon discontinued because of side effects. HU was restarted, but in December 2002 the patient deceased due to disease progression.

Ligation-mediated polymerase chain reaction and sequencing

To determine the DNA breakpoint region of the *µ-BCR-ABL* rearrangement in this CML as well as in a previously reported e19a2-positive AML and the e19a2-positive AR230 cell line, an LM-PCR was performed.[3-5] DNA (1 µg) was digested with 30 U blunt end restriction enzymes (*Dra*I, *Pvu*II, *Hin*cII, and *Stu*I). To both ends of the restriction fragments, 25 µM of adaptor DNA was ligated.[5] The ligation

Table 1. Sequences (5'–3' sequence) of the primers used in this study.

Primers used for RT-PCR analysis	
BCR-e19	TTC GGA GTC AAG ATT GCT GTG
ABL-a3 (B and E3')	see BIOMED-1 report (ref. 1)
Primers used for first round LM-PCR	
BCR-LM1	GGA GGT GGG CAT CTA CCG
AP1	see Przybylski *et al.* (ref. 5)
Primers used for second round LM-PCR	
BCR-LM2	CGG ACA TCC AGG CAC TGA A
AP2	see Przybylski *et al.* (ref. 5)
Primers used for sequencing	
*µ*BCR-sCML	CCT TCC TGC TAT CAG ACG AC
*µ*BCR-sAML	CTT CTT TGG GGC ACA CAC
*µ*BCR-sAR230	CCA GGA CCC CTA GAA TGC
Primers used for confirmation PCR	
*µ*BCR-cCML	GGG ATG GTG GGA GAC TCA
*µ*BCR-cAML	CCA GGA CCC CTA GAA TGC
*µ*BCR-cAR230	GCA GGG GTG GTT GCT ATG
ABL-cCML	GGC CCT TTC ATT CAA CG
ABL-cAML	CCC GAC CAG TGG ATG ATG
ABL-cAR230	TTC TGG AAT GGC TGG TAA AC

products were subjected to two PCRs, essentially performed as described before.[5] Briefly, a 50 µl reaction contained 1 µl of ligation products, 10 pmol of AP1 primer and BCR-LM1 primer (Table 1), 10 mM dNTP (Amersham Biosciences Corp., Piscataway, New Jersey, USA), 2U rTth (Applied Biosystems, Foster City, CA, USA), and 1.5 mM $Mg(OAc)_2$. Subsequently, a second round PCR was performed using 1 µl of the first-round PCR product and 10 pmol of the internal AP2 and BCR-LM2 primers (Table 1). Specific primers (Table 1) were used for direct sequencing of the gel-extracted bands (Zymoclean gel DNA recovery kit, Zymo Research, CA, USA). The presence of µ-*BCR-ABL* rearrangements was confirmed by PCR analysis performed on undigested genomic DNA using *µ*-BCR- and ABL-specific primers (Table 1).

RESULTS

DNA was digested with *Dra*I, *Pvu*II, *Hin*cII, and *Stu*I restriction enzymes: ten restriction sites were found in the germline *BCR* sequence between exons 18 and 21 (Figure 1A). The first-round LM-PCR identified germline PCR products of 253, 1992, 1679, and 1317 bp in *Dra*I, *Pvu*II, *Hin*c II, and *Stu*I digests, respectively, while the second-round LM-PCR gave germline PCR products of 206, 1945, 1632, and 1270 bp, respectively. In the AR230 cell line, an additional non-germline PCR product of 1014 bp was identified after the second PCR of *Hin*cII digested and amplified DNA, while nested LM-PCR of the CML patient and the AML patient revealed additional PCR products of 1469 and 1086 bp, respectively, in *Pvu*II digested DNA (Figure 1B). Direct sequencing of the gel-extracted bands identified all three genomic *µ-BCR-ABL* fusion sites (Table 2). At the fusion site of *BCR* and *ABL* intronic sequences, no insertions or duplications of nucleotides were found.

The three *µ*-BCR breaks occurred in a 393 bp stretch of intron 19. In the CML patient, almost the entire sequence of intron 19 was preserved, with only six nucleotides deleted. The BCR breakpoint in AR230 was also located in the

Figure 1.
(A) Schematic representation of the BCR gene. In total, 10 restriction sites of DraI (D), PvuII (P), HincII (H), and StuI (S) in the BCR gene between exons 18 and 21 are shown. The bold arrows show the location of the *µ*-BCR DNA breakpoints in the AML patient, the CML patient, and the AR230 cell line (Genbank accession code: U07000).
(B) Agarose gel electroforesis of amplified DNA after nested LM-PCR. Results of germline controls (gc), the AML patient, the CML patient, and the AR230 cell line are shown. Germline bands of 1945 and 1632 bp were found in *Pvu*II and *Hin*cII digested DNA, respectively. Additional bands (encircled) differing in size from the germline control were seen in *Pvu*II digested DNA of the AML patient (1086 bp) and the CML patient (1469 bp), and in *Hin*cII digested DNA of the AR230 cell line (1014 bp). *100 bp ladder; ** λ/*Pst*I DNA ladder.
(C) Schematic representation of the *ABL* gene showing the approximate location of the breakpoint regions in the first intron of the *ABL* gene. A, B and C indicate breakpoint regions, respectively, 30 kb ± 5, 100 kb ± 13, and 135 kb ± 8 downstream from exon 1b, involved in M-BCR rearrangements.[8] D indicates the *ABL* breakpoint region involved in the three *µ*-BCR rearrangements, which is located 162 ± 12 kb downstream from exon 1b. The bold arrows show the precise location of the *ABL* DNA breakpoints in the AR230 cell line, the CML patient and the AML patient (Genbank accession code: U07563).

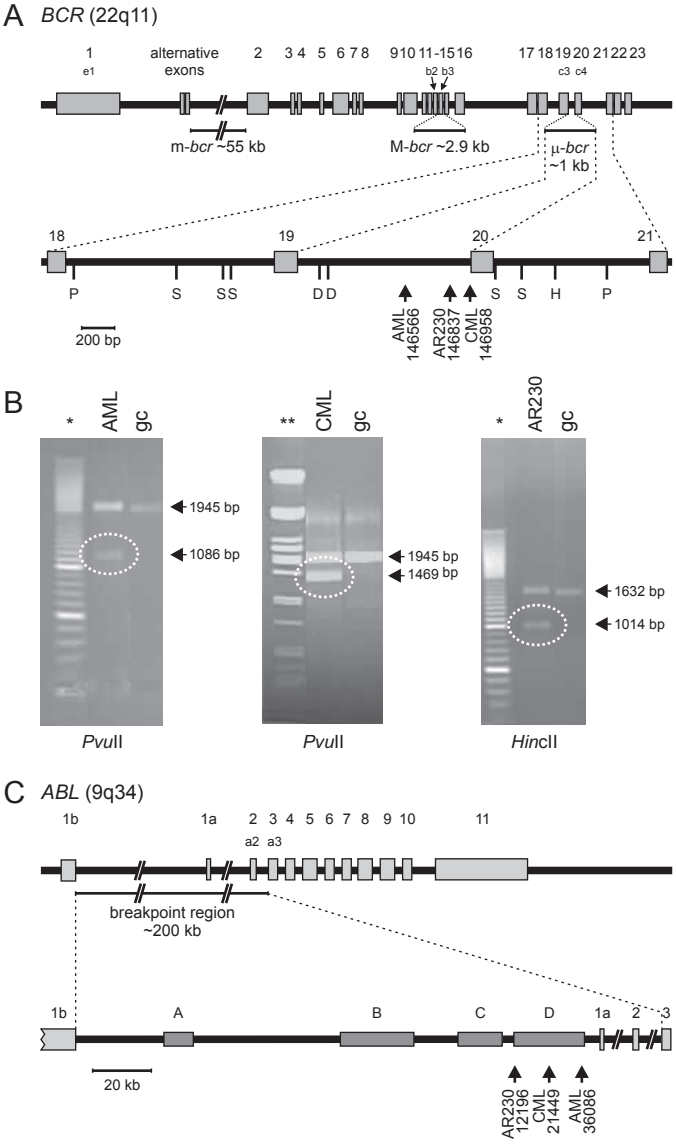

A BCR (22q11)
1
e1
alternative exons
2
3 4 5 6 7 8
9 10 11-15 16
b2 b3
17 18 19 20 21 22 23
c3 c4
m-bcr ~55 kb
M-bcr ~2.9 kb
µ-bcr ~1 kb
18
19
20
21
P
S
S S
D D
S
S
H
P
200 bp
AML 146566
AR230 146637
CML 146958
B
*
AML
gc
1945 bp
1086 bp
PvuII
**
CML
gc
1945 bp
1469 bp
PvuII
*
AR230
gc
1632 bp
1014 bp
HincII
C ABL (9q34)
1b
1a
2 3 4 5 6 7 8 9 10
a2 a3
11
breakpoint region ~200 kb
1b
A
B
C
D
1a
2
3
20 kb
AR230 12196
CML 21449
AML 36086

Table 2. *μ-BCR-ABL* DNA breakpoint sequences for the three e19a2 junctions amplified by LM-PCR.

	μ-*BCR-ABL*
CML	CCGCTGCATTTCCGTGC**TCT**CATCCTGCCGGGCGCGATGG
AML	TGTAAGCACCAATGGCCAAT**T**AATGACATCCTGGCCGAGAA
AR230	TGGGAGGAGTCAGATGAGCT**G**TATGGCCCAGTCAAGTTGAC

The BCR sequence is underlined. Overlapping sequences of BCR and ABL genes are depicted bold.

downstream part of intron 19, while in the AML patient, the break occurred more towards the middle of intron 19 (Figure 1A).

Breakpoints in the *ABL* gene were distributed over ~24 kb at the 3'end of the first *ABL* intron (Figure 1C). In the CML patient, the *ABL* breakpoint occurred within an *Alu* element, and in the AR230 cell line *Alu* repeats were located within 400 bp at both sides of the breakpoint. No repeats were found in the *μ-BCR* breakpoint region.

DISCUSSION

This is the first study in which LM-PCR was used to identify the exact DNA breakpoints in the *ABL* and *BCR* genes in e19a2 junctions. The low incidence of e19a2 junctions may partly be caused by the relatively short length of intron 19 (~1 kb) as compared with m-BCR (~55 kb) and M-BCR (~2.9 kb). Currently, primary factors that determine the preferential breakage sites in the *BCR* and *ABL* genes are unknown. It has been proposed that high densities of repetitive DNA, such as *Alu* elements, could provide hot spots for homologous recombination and mediate chromosomal translocation. The characterization of genomic DNA breakpoints in CML with *BCR-ABL* translocations has been reported.[6,7] In some cases, the *BCR* gene preferentially recombined with *Alu* elements; however, no unique pattern explained all DNA breaks. In our study, the *μ-BCR* recombined with regions close to or within *Alu* elements of the *ABL* gene in two out of three cases.

Conflicting data are reported with regard to the breakpoint sites in the *ABL* gene in CML. Nonrandom chromosomal breakpoints within the first intron of *ABL* have been suggested, with three breakpoint cluster regions downstream from exon 1b.[8] However, others reported equally dispersed breaks over intron 1b without any clustering.[9] The *ABL* breaks in our three e19a2 junctions were scattered over a ~24 kb region at the 3' part of intron 1b. During transcription exon 1a is probably spliced out, resulting in the *BCR* exon 19/*ABL* exon a2 mRNA junction.

Transcription of the *p230 BCR-ABL* gene in the great majority of patients with N-CML is extremely low: the level of p230$^{BCR-ABL}$ transcripts in untreated N-CML patients was similar to the level of p210 $^{BCR-ABL}$ transcripts in typical CML patients in cytogenetic remission after interferon-α treatment.[10] This low transcription level of

the *p230*$^{BCR-ABL}$ gene in most patients may hamper the use of RT-PCR at diagnosis and particularly during follow-up, which can be overcome by using genomic DNA breakpoint fusion sites as PCR targets. However, because the DNA breakpoint fusion regions, particularly of the *ABL* gene, are scattered over large regions and differ in each patient, special methods are needed for identification of these DNA breakpoint fusion sites. In our study, we used an LM-PCR to identify the e19a2 fusion at the DNA level.

Patient-specific DNA breakpoint fusion sites may be attractive PCR targets for monitoring of minimal residual disease (MRD) by real-time quantitative PCR using an allele-specific reverse primer positioned at the breakpoint area of the fusion genes, in combination with a *µ*-BCR-specific forward primer and probe. DNA targets for disease monitoring have several advantages over RNA targets, including low degradation rate, easy quantification (as only one target per cell is present), and stability throughout the disease course.

In conclusion, using LM-PCR, we identified DNA sequences of three e19a2 junctions which may potentially be used as patient-specific DNA targets for monitoring of MRD. Although *Alu* elements might be involved in some *µ-BCR-ABL* rearrangements, further studies on a larger series of e19a2-positive leukemias are needed to better understand the mechanisms by which *µ-BCR-ABL* DNA breaks are mediated.

ACKNOWLEDGEMENTS

We are grateful to Prof. Dr. N. Blanckaert for his continuous support, to J.M. Wijkhuijs and D. Jacobs for their technical assistance and to W.M. Comans-Bitter for preparation of the figures. We thank Dr. H. Wada who kindly donated the AR230 cell line. This work was supported by a grant of the IGA MZ CR: NC/7560-3.

REFERENCES

1. Van Dongen JJM, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Sagli G, Gottardi E, Rambaldi A, Dotti G, Griesinger F, Parreira A, Gameiro P, Diaz MG, Malec M, Langerak AW, San Miguel JF, Biondi A. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. *Leukemia* 1999; **13**: 1901-1928.
2. Pane F, Frigeri F, Sindona M, Luciano L, Ferrara F, Cimino R, Meloni G, Saglio G, Salvatore F, Rotoli B. Neutrophilic-chronic myeloid leukemia: a distinct disease with a specific molecular marker (*BCR/ABL* with c3/a2 junction). *Blood* 1996; **88**: 2410-2414.
3. Haskovec C, Ponzetto C, Polak J, Maritano D, Zemanova Z, Serra A, Michalova K, Klamova H, Cermak J, Saglio G. P230 BCR/ABL protein may be associated with an acute leukaemia phenotype. *Br J Haematol* 1998; **103**: 1104-1108.

4. Wada H, Mizutani S, Nishimura J, Usuki Y, Kohsaki M, Komai M, Kaneko H, Sakamoto S, Delia D, Kanamaru A, Kakishita E. Establishment and molecular characterization of a novel leukemic cell line with Philadelphia chromosome expressing p230 BCR/ABL fusion protein. *Cancer Res* 1995; **55**: 3192-3195.
5. Przybylski GK, Dik WA, Wanzeck J, Grabarczyk P, Majunke S, Martin-Subero I, Siebert R, Dolken G, Ludwig W, Verhaaf B, Van Dongen JJM, Schmidt CA, Langerak AW. Disruption of the BCL11B gene through inv14(q11.2;q32.31) results in expression of *BCL11B-TCRD* fusion transcripts and is associated with absence of wild-type *BCL11B* transcripts in T-ALL. *Leukemia* 2005; **19**: 201-208.
6. Jeffs AR, Benjes SM, Smith TL, Sowerby SJ, Morris CM. The *BCR* gene recombines preferentially with *Alu* elements in complex *BCR-ABL* translocations of chronic myeloid leukaemia. *Hum Mol Genet* 1998; **7**: 767-776.
7. Zhang JG, Goldman JM, Cross N. Characterization of genomic *BCR-ABL* breakpoints in chronic myeloid leukaemia by PCR. *Br J Haematol* 1995; **90**: 138-146.
8. Jiang XY, Trujillo JM, Liang JC. Chromosomal breakpoints within the first intron of the *ABL* gene are nonrandom in patients with chronic myelogenous leukemia. *Blood* 1990; **76**: 597-601.
9. Waller CF, Dennebaum G, Feldmann C, Lange W. Long-template DNA polymerase chain reaction for the detection of the *BCR/ABL* translocation in patients with chronic myelogenous leukaemia. *Clin Cancer Res* 1999; **5**: 4146-4151.
10. Verstovsek S, Lin H, Kantarjian H, Saglio G, De Micheli D, Pane F, Garcia-Manero G, Intrieri M, Rotoli B, Salvatore F, Guo JQ, Talpaz M, Specchia G, Pizzolo G, Liberati AM, Cortes J, Quackenbush RC, Arlinghaus RB. Neutrophilic-chronic myeloid leukemia: low levels of p230 *BCR-ABL* mRNA and undetectable p230 BCR-ABL protein may predict indolent course. *Cancer* 2002; **94**: 2416-2425.

PART 3

Clinical applications of molecular and flow cytometric diagnostics in myeloid leukemias

CHAPTER 3.1

Clinical applications of molecular and flow cytometric diagnostics in detecting minimal residual disease and evaluating therapy efficacy in myeloid leukemia

1. CLASSICAL APPLICATIONS

MRD studies in myeloid leukemia using molecular as well as flow cytometric approaches have shown that monitoring of residual disease may help to assess the efficacy of treatment and enables the identification of particularly patients at high risk of relapse.[1-3]

The treatment effectiveness of new therapeutics (e.g. imatinib mesylate (IM)) targeting the molecular base of the disease, can accurately be monitored by real-time PCR. In Chapter 3.4 is shown how the currently available molecular techniques can provide inside into treatment effectiveness of IM in advanced CML after allogeneic bone marrow transplantation.

The kinetics of tumor load reduction during therapy as well as the increase of tumor cells at the time of relapse can be monitored by RQ-PCR analysis of fusion gene transcripts both in bone marrow (BM) and in peripheral blood (PB) samples. Comparing the RQ-PCR results of paired PB-BM samples is essential to evaluate the possibility of safely replacing BM by PB for PCR-MRD monitoring in clinical settings. As the regular collection of BM aspirates is a rather traumatic experience, especially in children, it would be beneficial if BM sampling could be replaced by PB sampling. In Chapter 3.3 we focused on paired samples of *CBFB-MYH11* positive AML.

2. SPECIFIC APPLICATIONS

In addition to the “classical” clinical applications of molecular and flow cytometric techniques for MRD detection and evaluating therapy efficacy in myeloid leukemia, both techniques can be applied for other purposes as well (Table 1). In Chapter 3.2 is shown how several molecular and flow cytometric techniques can be used to confirm or exclude the common origin of two phenotypically different malignancies in the same patient.

Flow cytometric assessment of the expression of certain antigens (e.g. CD33) on leukemic blast cells is currently used to guide therapeutic options with antibody targeted therapy (e.g. gemtuzumab ozogamicin (GO)). Only 30% of CD33+ AML patients enter a complete remission using GO; several mechanisms of GO resistance and treatment failure have been proposed. We investigated whether a high circulating CD33-antigen burden compromises the drug delivery to the BM, thereby limiting the efficacy of GO treatment in AML patients (Chapter 3.5).

Early diagnosis of AML in monozygotic twins and evaluation of Guthrie cards

The concordance of acute leukemia in infant or childhood monozygote twins has been recognized to be extraordinarily high.[4] The likely explanation is blood

Table 1. Clinical applications of molecular and flow cytometric techniques for MRD detection and evaluating therapy efficacy in AML.

Classical applications
- identification of AML patients at high risk of relapse based on the MRD-levels at specific time points during therapy
- evaluation of the treatment effectiveness in CML after allogeneic bone marrow transplantation and recognition of early relapse to guide therapeutic interventions (e.g. donor lymphocyte infusions)
- early detection of molecular relapse in APL to guide therapeutic interventions
Specific applications
- evaluation of Guthrie cards to demonstrate the prenatal origin in infant and childhood AML
- evaluation of Guthrie cards and blood samples of monozygotic twins in case of AML diagnosis (early diagnosis in the second twin and investigation of clonal relationship between the AML cells in both twins)
- assessment of CD33 expression on leukemic blast cells to guide therapeutic options with antibody targeted therapy
- evaluation of autologous stem cell products to guide purging decisions
- detection of bone marrow involvement during 'isolated' extramedullary AML (skin, CNS or testicular infiltration)
- assessment of a common origin of two phenotypically different malignancies in the same patient

chimerism: initiation of leukemia in one twin *in utero*, is followed by a spread to the co-twin by the vascular anastomoses that commonly exist within monochorionic placenta.[5] The intraplacental "metastasis" is endorsed by the demonstration of the presence of clonotypic sequences in archived Guthrie cards of both twins.[6] Clonal markers used in twin leukemia studies have been fusion genes and/or Ig/TCR gene rearrangements.[7-9] As concordant AML can develop at different ages[10,11], using molecular or flow cytometric techniques, it is possible to detect or exclude the presence of the AML clone in the unaffected monozygotic twin.

Guthrie cards have also been used to demonstrate the prenatal origin in infant and childhood acute leukemia. Recently in pediatric AML, MRD-PCR analyses of Guthrie cards of childhood *AML1-ETO* + AML patients showed the presence of the *AML1-ETO* fusion genes at birth in a substantial number of patients.[12]

Evaluation of MRD in autologous stem cell products to guide purging decisions in AML

A major drawback of the reinfusion of autologous BM or peripheral blood stem cell (PBSC) products is its possible contamination with residual malignant cells. The presence of MRD in the graft may contribute to relapse.[13] Studies showed that AML patients, who were autografted in first remission with purged stem cells, had a lower relapse rate and a longer leukemia-free survival compared to patients receiving unpurged transplants.[14] A major limitation of applying purging concerns the high toxicity of the purging procedure for normal progenitors/stem cells, with the

potential danger of increased morbidity and/or toxicity. However, the MRD frequency in PBSC products is predictive for duration of relapse free survival.[15] Using flow cytometric immunophenotyping, it becomes feasible to measure the MRD burden in the PBSC products based on leukemia-associated phenotypes established at AML diagnosis. A recent study proposed an MRD-based model that could be used to guide the decision whether or not to purge PBSC products.[16]

Evaluation of bone marrow involvement during "isolated" extramedullary AML

Extramedullary manifestations of AML include skin and gingival infiltrates, involvement of central nervous system (CNS), and testicular infiltration. Skin infiltration in AML patients usually occurs at presentation with BM disease. However, skin disease can precede BM infiltration up to 6 months (aleukemic leukemia cutis)[17,18] and skin infiltration may be a first sign of relapse, even if the skin was not involved at initial diagnosis.

Testicular infiltration in AML is rare and occurs predominantly in myelomonocytic or monoblastic leukemia. Cases of AML mimicking a primary testicular neoplasm have been reported.[19] The majority of patients subsequently progress to overt AML, but testicular infiltration can precede an overt AML with a median duration of 10.5 months.[20] Testicular relapses of AML can occur simultaneous with a BM relapse in 1-2% of patients,[21] but also "isolated" testicular relapses in the absence of systemic relapse have been reported; most of these patients experienced however BM relapse within 2 months after the recognition of the testicular relapse.[21] A testicular biopsy showing infiltration of the testicular tissue with malignant cells of myeloid origin should be considered as a harbinger of systemic leukemia.

Both flow cytometric and molecular techniques can be used to recognize sub-microscopic BM involvement in cases of primary or relapsed "isolated" extramedullary AML.

REFERENCES

1. San Miguel JF, Martinez A, Macedo A, Vidriales MB, Lopez-Berges C, Gonzalez M, Caballero D, Garcia-Marcos MA, Ramos F, Fernandez-Calvo J, Calmuntia MJ, Diaz-Mediavilla J, Orfao A. Immunophenotyping investigation of minimal residual disease is a useful approach for predicting relapse in acute myeloid leukemia patients. *Blood* 1997; **90**: 2465-2470.
2. Leroy H, De Botton S, Grardel-Duflos N, Darre S, Leleu X, Roumier C, Morschhauser F, Lai JL, Bauters F, Fenaux P, Preudhomme C. Prognostic value of real-time quantitative PCR (RQ-PCR) in AML with t(8;21). *Leukemia* 2005; **19**: 367-372.
3. Buonamici S, Ottaviani E, Testoni N, Montefusco V, Visani G, Bonifazi F, Amabile M, Terragna C, Ruggeri D, Piccaluga PP, Isidori A, Malagola M, Baccarani M, Tura S, Martinelli G. Real-time quantitation of minimal residual disease in inv(16)-positive acute myeloid leukemia may indicate risk for clinical relapse and may identify patients in a curable state. *Blood* 2002; **99**: 443-449.

4. Macmahon B, Levy MA. Prenatal origin of childhood leukaemia. Evidence from twins. *N Engl J Med* 1964; **270**: 1082-1085.
5. Clarkson BD, Boyse EA. Possible explanation of the high concordance for acute leukaemia in monozygotic twins. *Lancet* 1971; **1**: 699-701.
6. Gale KB, Ford AM, Repp R, Borkhardt A, Keller C, Eden OB, Greaves MF. Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. *Proc Natl Acad Sci USA* 1997; **94**: 13950-13954.
7. Ford AM, Pombo-de-Oliveira MS, McCarthy KP, MacLean JM, Carrico KC, Vincent RF, Greaves M. Monoclonal origin of concordant T-cell malignancy in identical twins. *Blood* 1997; **89**: 281-285.
8. Ford AM, Bennett CA, Price CM, Bruin MC, Van Wering ER, Greaves M. Fetal origins of the *TEL-AML1* fusion gene in identical twins with leukemia. *Proc Natl Acad Sci USA* 1998; **95**: 4584-4588.
9. Wiemels JL, Ford AM, Van Wering ER, Postma A, Greaves M. Protracted and variable latency of acute lymphoblastic leukemia after *TEL-AML1* gene fusion *in utero*. *Blood* 1999; **94**: 1057-1062.
10. Kempski H, Mensa-Bonsu KA, Kearney L, Jalali GR, Hann I, Khurshid M, Greaves M. Prenatal chromosomal diversification of leukemia in monozygotic twins. *Genes Chromosomes Cancer* 2003; **37**: 406-411.
11. Richkind KE, Loew T, Meisner L, Harris C, Wason D. Identical cytogenetic clones and clonal evolution in pediatric monozygotic twins with acute myeloid leukemia: presymptomatic disease detection by interphase fluorescence in situ hybridization and review of the literature. *J Pediatr Hematol Oncol* 1998; **20**: 264-267.
12. Wiemels JL, Xiao Z, Buffler PA, Maia AT, Ma X, Dicks BM, Smith MT, Zhang L, Feusner J, Wiencke J, Pritchard-Jones K, Kempski H, Greaves M. *In utero* origin of t(8;21) *AML1-ETO* translocations in childhood acute myeloid leukaemia. *Blood* 2002; **99**: 3801-3805.
13. Brenner MK, Rill DR, Moen RC, Krance RA, Mirro J Jr, Anderson WF, Ihle JN. Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* 1993; **341**: 85-86.
14. Gorin NC, Aegerter P, Auvert B, Meloni G, Goldstone AH, Burnett A, Carella A, Korbling M, Herve P, Maraninchi D, *et al*. Autologous bone marrow transplantation for acute myelocytic leukemia in first remission: a European survey of the role of marrow purging. *Blood* 1990; **75**: 1606-1614.
15. Feller N, Van der Pol MA, Van Stijn A, Weijers GW, Westra AH, Evertse BW, Ossenkoppele GJ, Schuurhuis GJ. MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. *Leukemia* 2004; **18**: 1380-1390.
16. Feller N, Jansen-van der Weide MC, Van der Pol MA, Westra GA, Ossenkoppele GJ, Schuurhuis GJ. Purging of peripheral blood stem cell transplants in AML: a predictive model based on minimal residual disease burden. *Exp Hematol* 2005; **33**: 120-130.
17. Baer MR, Barcos M, Farrell H, Raza A, Preisler HD. Acute myelogenous leukemia with leukemia cutis. Eighteen cases seen between 1969 and 1986. *Cancer* 1989; **63**: 2192-2200.
18. Wilkins R, Janes S. Aleukaemic leukaemia cutis: case report and review of the literature. *Clin Lab Haematol* 2004; **26**: 73-75.
19. McIlwain L, Sokol L, Moscinski LC, Saba HI. Acute myeloid leukemia mimicking primary testicular neoplasm. Presentation of a case with review of literature. *Eur J Haematol* 2003; **70**: 242-245.
20. Neiman RS, Barcos M, Berard C, Bonner H, Mann R, Rydell RE, Bennett JM. Granulocytic sarcoma: a clinicopathologic study of 61 biopsied cases. *Cancer* 1981; **48**: 1426-1437.
21. Shaffer DW, Burris HA, O'Rourke TJ. Testicular relapse in adult acute myelogenous leukemia. *Cancer* 1992; **70**: 1541-1544.

CHAPTER 3.2

An inv(16)(p13q22) positive acute myeloid leukemia relapsing as acute precursor B-cell lymphoblastic leukemia

Nancy Boeckx[1], Vincent H.J. van der Velden[2], Marc Boogaerts[3], Anne Hagemeijer[4], Peter Vandenberghe[4], Jacques J.M. van Dongen[2]

[1]Department of Laboratory Medicine, Laboratory of Hematology, University Hospital Gasthuisberg, Leuven, Belgium
[2]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, The Netherlands
[3]Department of Internal Medicine, Hematology, University Hospital Gasthuisberg, Leuven, Belgium
[4]Department of Human Genetics, KU Leuven, Leuven, Belgium

Adapted from letter. Haematologica 2004; 89(8): ECR28.

ABSTRACT

We describe a case of a 38-year old male with inv(16)(p13q22) positive acute myeloid leukemia (AML) with eosinophilia, relapsing after a molecular remission of almost three years. Remarkably, the leukemia at relapse was identified as a precursor-B-cell acute lymphoblastic leukemia (B-ALL) by cytology and immunophenotyping, but was inv(16)(p13q22) positive as revealed by interphase FISH, FICTION analysis, and real-time quantitative PCR. Analysis of immunoglobulin and T-cell receptor genes showed a bi-allelic D_H2-J_H rearrangement at relapse, but not at diagnosis. These findings indicate a myeloid to lymphoid lineage switch from an inv(16)(p13q22) positive leukemia and show that *IGH* gene rearrangements can occur in the presence of *CBFB-MYH11* fusion transcripts.

INTRODUCTION

Lineage switch is a rare event in acute leukemia at relapse, and usually concerns a therapy-related switch from ALL to AML late in the disease course.[1] Conversion of AML to ALL has only been reported in five cases, with a rather short time from diagnosis to conversion and without stable genotypic aberrations.[2] Here, we report clinical and laboratory features of an adult patient with inv(16)(p13q22) positive AML with eosinophilia, who relapsed with inv(16)(p13q22) positive precursor-B-ALL.

MATERIALS AND METHODS

Case report

A 38-year old man was admitted to the hospital in February 1998 with a history of fatigue. Laboratory studies revealed a hemoglobin of 4.87 g/dl, a white blood count of 14.8 x 10^9/l with 41% blasts, and a platelet count of 31 x 10^9/l. A bone marrow (BM) aspirate contained 68% blasts and 15% aberrant eosinophils (Figure 1). Based on morphology and cytochemical stainings, it was classified as an AML-M4Eo according to the French-American-British classification. Cytogenetic analysis showed 46,XY,inv(16)(p13q22) and real-time quantitative PCR (RQ-PCR) analysis confirmed a *CBFB-MYH11* type A fusion gene transcript. With intensive chemotherapy according to an AML treatment protocol, he achieved molecular remission. After a molecular BM relapse in August 1999, he underwent an allogeneic stem cell transplantation (SCT) and remained in molecular remission for almost three years. In August 2002, RQ-PCR became positive again for the *CBFB-MYH11* type A fusion gene transcript, with a BM relapse of a precursor-B-ALL ($CD10^+CD19^+$) four months later. Cytogenetic analysis was not done. A *CBFB-MYH11* fusion gene was found by RQ-PCR analysis. Re-induction therapy and donor lymphocyte infusions were given, but in May 2003 the patient relapsed again with inv(16)(p13q22) positive B-ALL. He deceased in July 2003 because of multiple organ failure within two weeks after a second allogeneic SCT.

BM and PB samples obtained at diagnosis, during follow-up and relapse were used for flow cytometric and molecular investigations.

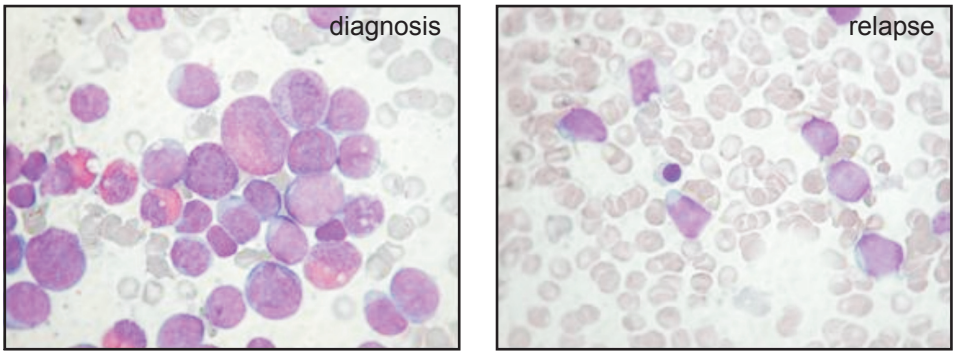


Figure 1.
Morphological features (May Grünwald Giemsa staining) at presentation and relapse. At diagnosis (left), the BM contained 68% blasts and 15% aberrant eosinophils. The relapse (right) BM showed 49% blast cells and 0.5% eosinophils.

Flow cytometric immunophenotyping

Lysed whole blood or BM was stained using directly conjugated monoclonal antibodies. The specificity and source of the antibodies are summarized in Table 1. At diagnosis, measurements were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) by double labelling and blast cells were gated by their FSC-SSC characteristics. At relapse, cells were analysed on a FACSCalibur (Becton Dickinson) and gated by their CD45-SSC characteristics.

Real-time quantitative polymerase chain reaction

BM and PB samples were analysed by RQ-PCR. RNA was isolated from frozen mononuclear cells using the QIAamp Blood Kit (Qiagen, USA). The cDNA synthesis (40 μl) was performed using 200 units of M-MLV Reverse Transcriptase (Gibco BRL), 25 μM Random Hexamers (Amersham Pharmacia Biotech, USA) and 2 μg RNA. A 25 μl RQ-PCR reaction mixture contained TaqMan Universal PCR Master Mix 2x (Applied Biosystems, Foster City, CA, USA), 300 nM primers, 200 nM TaqMan probes and 5 μl of cDNA. Primer-probe sets were published elsewhere.[3-4] Detection of PCR products was based on TaqMan technology using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Standard curves were established using serial dilutions of *CBFB-MYH11* type A or β-glucuronidase (*GUS*) plasmids (Ipsogen, Marseille, France). The copy numbers of the *CBFB-MYH11* fusion gene transcript were normalized to *GUS*.

Analysis of immunoglobulin and T-cell receptor gene rearrangements

DNA was isolated from frozen mononuclear cells and PCR analysis of *IGH*, *IGK*-Kde, *TCRG*, and *TCRD* gene rearrangements was performed as described previously.[5] For detection of Vδ2-Jα rearrangements, 7 multiplex tubes, each containing one Vδ2 primer and 8 to 10 Jα primers, were used. *TCRB* rearrangements were analysed using BIOMED-2 primers.[6-7] The obtained PCR products were subjected to heteroduplex analysis and clonal rearrangements were subsequently sequenced using the Big Dye-terminator cycle sequencing kit and an ABI 377 automated sequencer (Applied Biosystems) as described previously.[8-9]

Chimerism studies

Short tandem repeats (STR) and the amelogenin locus were amplified using the AmpFLSTR SGM and Profiler Plus™ PCR Amplification Kits (Applied Biosystems, Lennik, Belgium), according to the

manufacturer's instructions. The PCR products were analysed on the ABI 3100 genetic analyser. For informative STRs, the height of the donor-derived allele was divided by the sum of the heights of donor and receptor alleles. The percentage donor chimerism is the average of this value for all informative STRs, multiplied by 100.

FISH and FICTION analysis

FISH (fluorescence *in situ* hybridization) was done on a relapse BM smear using the LSI CBFB dual color break apart probe for the region 16q22, as described by the manufacturer (Vysis Abbott Laboratories, IL, USA). 200 interphase nuclei were scored. FICTION (**f**luorescence **i**mmunophenotyping and interphase **c**ytogenetics as a **t**ool for the **i**nvestigation **o**f **n**eoplasms) using anti-CD10 (clone:W8E7, Becton Dickinson) and the LSI CBFB dual color break apart probe for the region 16q22 was done on a follow-up BM smear as described elsewere.[10]

RESULTS

At diagnosis, flow cytometric analysis showed $CD13^+CD33^+MPO^+$ myeloblasts co-expressing CD19 and with partial expression of TdT (Table 1, Figure 2), cytogenetic analysis showed 46,XY,inv(16)(p13q22) and RQ-PCR confirmed a *CBFB-MYH11* type A fusion transcript (Figure 3).

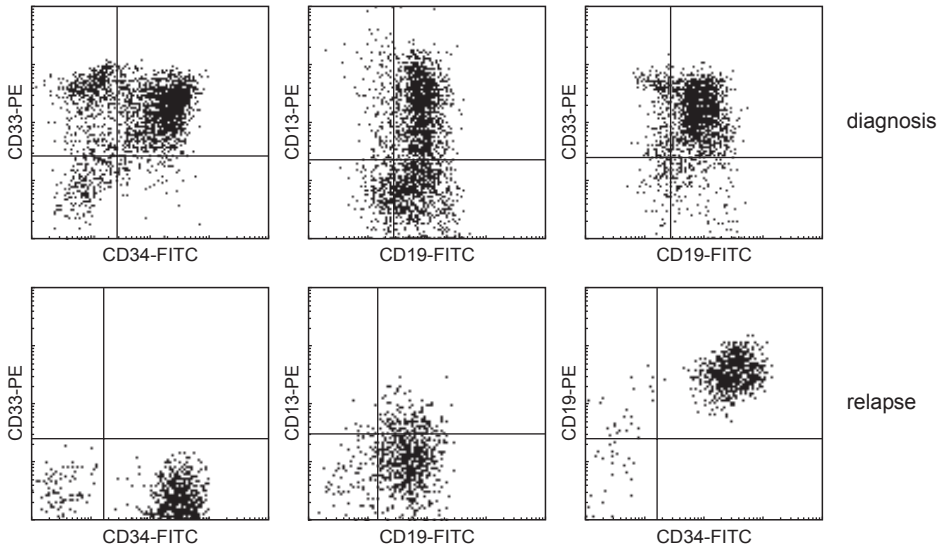


Figure 2.
Immunophenotypical features at presentation and relapse. Upper plots: diagnostic BM sample showing $CD13^+CD33^+CD34^+CD19^+$ myeloblasts. Lower plots: triple labelling of the relapse BM sample showing $CD13^-CD33^-CD34^+CD19^+$ B-lymphoblasts.

Table 1.

	Diagnosis	**Relapse (59 months)**
Cytomorphology	AML-M4Eo (FAB)	Acute leukemia
Percentage of blasts in BM	68 (67% on ANC)	49 (36% on ANC)
Percentage of eosinophils in BM	15	0.5
Cytochemistry		
Peroxidase	positive	negative
Immunophenotype		
Percentage of blast cells on ANC	51	10
	(% positivity within the gated blast cell population)	
CD34 (8G12)[a]	70	92
CD117 (95C3)[b]	48	0
TdT (HT-6)[c]	48	84
HLA-DR (L243)[a]	98	ND
CD13 (L138)[a]	58	14
CD15 (MMA)[a]	61	4
CD33 (P67.6)[a]	87	0
CD65 (VIM2)[d]	85	ND
MPO (MPO-7)[e]	80	0
CD10 (HI10a)[a]	0	87 (strong)
CD19 (4G7)[a]	80	87
CD20 (L27)[a]	0	25
CD24 (ALB9)[b]	1	64
CD2 (S5.2)[a]	0	ND
Immunogenotype		
Karyotype	46,XY,inv(16)(p13q22)	ND
RT-PCR	*CBFB-MYH11*, type A +	*CBFB-MYH11*, type A + *MLL-AF4* - *BCR-ABL* -
FISH (probe: CBFB)	ND	split signal in 15%
IGH gene	no rearrangement detected	$D_H2.2$-J_H6b (-2/12/-9) $D_H2.15$-J_H5b (-8/5/-4)
IGK gene	no rearrangement detected	no rearrangement detected
TCRD, *TCRG*, *TCRB* gene	no rearrangement detected	no rearrangement detected
DNA profile	100% receptor	100% receptor

ANC: all nucleated cells, ND: not done

[a] Beckton Dickinson, San Jose, CA, USA; [b] Immunotech/Coulter, Marseille, France; [c] IKT diagnostics, Uithoorn, The Netherlands; [d] Caltag, San Francisco, CA, USA; [e] DAKO, Glostrup, Denmark.

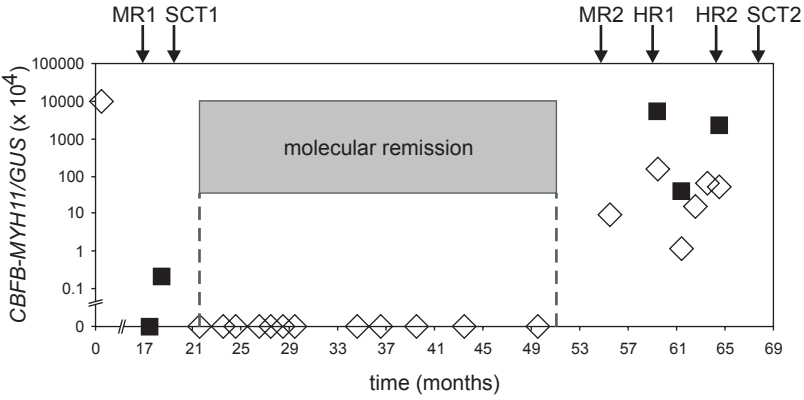


Figure 3.
RQ-PCR analysis of *CBFB-MYH11* transcripts at presentation and relapse. The normalized copy numbers of the *CBFB-MYH11* type A fusion transcripts are shown as a function of time. ◊ represent *CBFB-MYH11* fusion transcripts in PB, ■ represent *CBFB-MYH11* fusion transcripts in BM. MR: molecular relapse. HR: hematological-morphological relapse. SCT: stem cell transplantation.

At relapse, immunophenotypic analysis revealed a $CD10^+CD19^+$ precursor-B-ALL with 10% leukemic B-lymphoblasts and without leukemic myeloblasts (Table 1, Figure 2). High levels of *CBFB-MYH11* type A fusion transcripts were found by RQ-PCR (Figure 3), while *MLL-AF4* and *BCR-ABL* were not present. Chimerism studies identified a 100% recipient DNA profile. FISH analysis showed a split signal in 15% of 200 nuclei (Figure 4). Additional FICTION analysis identified CD10 positive mononuclear cells displaying a split signal indicative of inv(16)(p13q22) (Figure 4). CD10 negative cells had normal hybridisation signals. Split signals were detected exclusively in the CD10 positive cells.

PCR heteroduplex analyses of *IGH*, *IGK*-Kde, *TCRG*, *TCRB,* and *TCRD* gene rearrangements, was performed on diagnostic and relapse samples. No Ig/TCR gene rearrangements were found at diagnosis, but bi-allelic D_H2-J_H rearrangements were found at relapse (Table 1). Two patient specific D_H2-J_H RQ-PCR assays were developed and reached a sensitivity of $10^{-4}/10^{-5}$. RQ-PCR on the diagnostic AML showed no specific D_H2-J_H products.

DISCUSSION

Conversions from AML to ALL are extremely rare, with only three pediatric and two adult cases reported.[2] In these cases, the interval between diagnosis and conversion was rather short (range: 10-13 months), in contrast to our patient where

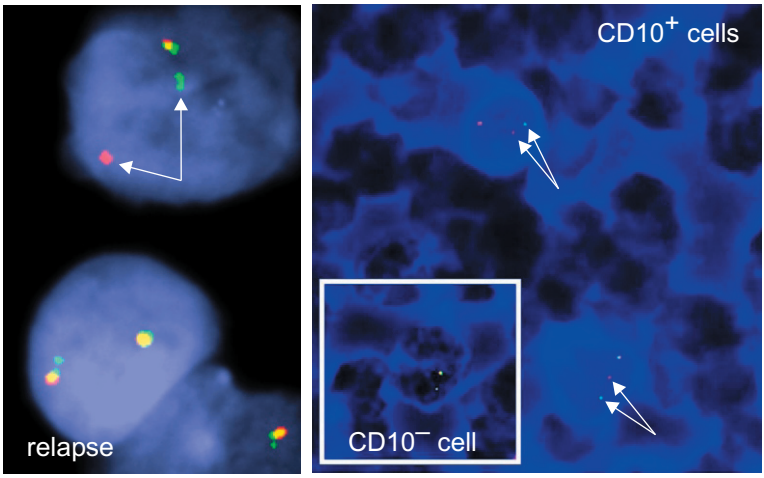


Figure 4.
(Left) FISH analysis on a relapse BM smear. On the normal chromosome 16, the green and red signal are colocalized yielding a yellow signal. Arrows show split signals indicative of inv(16)(p13q22).
(Right) FICTION analysis on a follow-up bone marrow smear of May 2003 with 7% residual leukemic B-lymphoblast. CD10 positive (intense blue staining) mononuclear cells (lymphoblasts) show split signals (arrows) indicative of inv(16)(p13q22). CD10 negative cells (a neutrophil band form is shown) have normal hybridisation signals. Split signals were detected exclusively in the CD10 positive cells.

the interval between AML and ALL was 59 months. Moreover, the clonal relationship between diagnosis and relapse was not reported in these published patients. The present patient is exceptional because he developed a precursor-B-ALL that was clonally related to his previous AML, as demonstrated most convincingly by FICTION analysis. To our knowledge, this is the first patient with *CBFB-MYH11*-positive precursor-B-ALL. It has to be assumed that the malignant clone withstood chemotherapy and allogeneic transplantation, but stayed under the detection limit (10^{-5}) of our current RQ-PCR assay during almost three years.

Sixteen cases of donor cell leukemia (DCL) after transplantation for leukemia have been reported, including one case with a t(9;22)(q34;q11) in recipient and donor leukemia.[11] A longer interval between transplantation and relapse seems to favour the possibility that relapse originates from donor cells. In our case, molecular studies excluded the possibility of DCL.[12]

The AML cells present at diagnosis were CD19 positive, a marker frequently correlated with the presence of t(8;21)(q22;q22). Previous studies showed a significant association between positivity for B-cell markers and the presence of Ig/TCR rearrangements in AML.[9] However, no Ig/TCR gene rearrangements were found in the AML at diagnosis, whereas bi-allelic incomplete *IGH* gene rearrangements were observed in the precursor-B-ALL at relapse. Probably,

activation of recombination activating gene products and TdT was induced when the leukemic cells switched from myeloid to B-cell lineage, resulting in the *IGH* gene rearrangements. The presence of *CBFB-MYH11* fusion transcripts apparently did not block these rearrangements. However, the sole presence of incomplete *IGH* gene rearrangements in the precursor-B-ALL reflects a very immature immunogenotype and is found <1% of primary precursor-B-ALL, suggesting that the original myeloid origin and/or the presence of *CBFB-MYH11* fusion transcripts might affect the Ig/TCR gene rearrangement process.[13]

The presented patient is exceptional because he developed a clonally related precursor-B-ALL after AML. Apparently, smouldering malignant cells can be present during many years and these cells might have the potential of changing their immunophenotype. Alternatively, the *CBFB-MYH11* aberration might have occurred as a first hit, with separate second hits resulting in the AML-M4Eo at first presentation and the precursor-B-ALL at second presentation. However, the precise mechanisms to fully explain our findings await further insights.

ACKNOWLEDGEMENTS

We are grateful to Prof. Dr. N. Blanckaert for his continuous support and to J. De Roover, M. Stul, P.G. Hart and B. Emanuel for their technical assistance. This work was supported by grant G-9.0224.99 from the Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen. P. Vandenberghe is a Clinical Investigator of the FWO Vlaanderen.

REFERENCES

1. Stass S, Mirro J, Melvin S, Pui CH, Murphy SB, Williams D. Lineage switch in acute leukemia. *Blood* 1984; **64**: 701-706.
2. Lounici A, Cony-Makhoul P, Dubus P, Lacombe F, Merlio JP, Reiffers J. Lineage switch from acute myeloid leukemia to acute lymphoblastic leukemia: report of an adult case and review of the literature. *Am J Hematol* 2000; **65**: 319-321.
3. Gabert J, Beillard E, Van der Velden VHJ, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cave H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, Gonzalez M, Viehmann S, Malec M, Saglio G, Van Dongen JJM. Standardization and quality control studies of “real-time” quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) of fusion gene transcripts for minimal residual disease detection in leukemia - A Europe Against Cancer Program. *Leukemia* 2003; **12**: 2318-2357.
4. Beillard E, Pallisgaard N, Van der Velden VHJ, Bi W, Dee R, Van der Schoot E, Delabesse E, Macintyre E, Gottardi E, Saglio G, Watzinger F, Lion T, Van Dongen JJM, Hokland P, Gabert J. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using ‘real-time’ quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. *Leukemia* 2003; **12**: 2474-2486.

5. Pongers-Willemse MJ, Seriu T, Stolz F, d'Aniello E, Gameiro P, Pisa P, Gonzalez M, Bartram CR, Panzer-Grumayer ER, Biondi A, San Miguel JF, Van Dongen JJM. Primers and Protocols for Standardized Detection of Minimal Residual Disease in Acute Lymphoblastic Leukemia Using Immunoglobulin and T Cell Receptor Gene Rearrangements and TAL1 Deletions as PCR targets: Report of the BIOMED-1 CONCERTED ACTION: Investigation of Minimal Residual Disease in Acute Leukemia. *Leukemia* 1999; **13**: 110-118.
6. Droese J, Langerak AW, Groenen PJ, Bruggemann M, Neumann P, Wolvers-Tettero IL, Van Altena MC, Kneba M, Van Dongen JJM. Validation of BIOMED-2 multiplex PCR tubes for detection of TCRB gene rearrangements in T-cell malignancies. *Leukemia* 2004; **9**: 1531-1538.
7. Van Dongen JJM, Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender L, Delabesse E, Davi F, Schuuring E, Garcia-Sanz R, Van Krieken JH, Droese J, Gonzalez D, Bastard C, White HE, Spaargaren M, Gonzalez M, Parreira A, Smith JI, Morgan GJ, Kneba M, Macintyre EA. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations. Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003; **17**: 2257-2317.
8. Langerak AW, Szczepanski T, Van der Burg M, Wolvers-Tettero IL, Van Dongen JJM. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. *Leukemia* 1997; **12**: 2192-2199.
9. Boeckx N, Willemse MJ, Szczepanski T, Van der Velden VHJ, Langerak AW, Vandekerckhove P, Van Dongen JJM. Fusion gene transcripts and Ig/TCR gene rearrangements are complementary but infrequent targets for PCR-based detection of minimal residual disease in acute myeloid leukemia. *Leukemia* 2002; **16**: 368-375.
10. Martin-Subero JI, Chudoba I, Harder L, Gesk S, Grote W, Novo FJ, Calasanz MJ, Siebert R. Multicolor-FICTION: expanding the possibilities of combined morphologic, immunophenotypic, and genetic single cell analyses. *Am J Pathol* 2002; **161**: 413-420.
11. Cooley LD, Sears DA, Udden MM, Harrison WR, Baker KR. Donor cell leukemia: report of a case occurring 11 years after allogeneic bone marrow transplantation and review of the literature. *Am J Hematol* 2000; **63**: 46-53.
12. Spinelli O, Giussani U, Borleri G, Lazzari M, Michelato A, Dotti G, Barbui T, Rambaldi A. Need for an accurate molecular diagnosis to assess the donor origin of leukemia relapse after allogeneic stem cell transplantation. *Haematologica* 2000; **85**: 1153-1157.
13. Van der Velden VHJ, Szczepanski T, Wijkhuijs AJM, Hart PG, Hoogeveen PG, Hop WCJ, Van Wering ER, Van Dongen JJM. Age-related patterns of immunoglobulin and T-cell receptor gene rearrangements in precursor-B-ALL: implications for detection of minimal residual disease. *Leukemia* 2003; **17**: 1834-1844.

CHAPTER 3.3

Quantification of *CBFB-MYH11* fusion gene levels in paired peripheral blood and bone marrow samples by real-time PCR

Nancy Boeckx[1,2], Jan De Roover[2], Vincent H.J. van der Velden[3], Johan Maertens[4], Anne Uyttebroeck[5], Peter Vandenberghe[2,6], Jacques J.M. van Dongen[3]

[1]Department of Laboratory Medicine, Laboratory of Hematology, University Hospital Gasthuisberg, Leuven, Belgium
[2]Center for Molecular Diagnostics, University Hospital Gasthuisberg, Leuven, Belgium
[3]Department of Immunology, Erasmus MC, Rotterdam, The Netherlands
[4]Department of Internal Medicine, Hematology, University Hospital Gasthuisberg, Leuven, Belgium
[5]Department of Pediatrics, University Hospital Gasthuisberg, Leuven, Belgium
[6]Department of Human Genetics, KU Leuven, Leuven, Belgium

Adapted from letter. Leukemia 2005; 19: 1988-1990.

ABSTRACT

The inv(16)(p13q22) chromosomal rearrangement is characterized by the presence of the *CBFB-MYH11* fusion gene transcript that can be used to monitor minimal residual disease (MRD). In this study, we evaluated the possibility to safely replace bone marrow (BM) sampling by peripheral blood (PB) sampling for MRD monitoring in *CBFB-MYH11*+ acute myeloid leukemia (AML). Therefore, we analyzed by real-time quantitative PCR the *CBFB-MYH11* transcripts in 128 PB and 67 BM samples, including 64 paired PB-BM samples from 10 different AML patients. Results showed a moderate correlation (r_s = 0.82) between the MRD levels in the follow-up PB-BM pairs; generally higher MRD levels were found in the BM samples than in the PB samples (ranges: 0.8 to 100 fold). Nevertheless, monitoring of *CBFB-MYH11* transcript levels in PB did have predictive value: firstly, MRD negative PB samples after the second course predicted a favorable prognosis with a long term remission. Secondly, MRD levels in the PB predicted the disease reoccurrence 2.5 to 4 months prior to the cytological BM relapse. Although further studies are needed, our preliminary data show that *CBFB-MYH11*+ AML can be monitored through the regular evaluation of PB at regular time points, thereby avoiding the multiple invasive BM punctures.

INTRODUCTION

Inv(16)(p13q22) and the related translocation t(16;16)(p13;q22) generate a *CBFB-MYH11* fusion gene transcript and are commonly found in acute myeloid leukemia (AML) M4Eo.[1] This recurrent chromosomal anomaly is considered a good risk marker, associated with a high complete remission rate and a long-term disease free survival.[2] Nevertheless, a considerable proportion of patients suffer from relapse. Analysis of minimal residual disease (MRD) may allow recognition of patients at high risk of relapse and/or may detect an upcoming relapse. Buonamici *et al*[3] used real-time quantitative PCR (RQ-PCR) analysis and suggested the existence of cut-off points in *CBFB-MYH11*+ AML beneath which durable remission is likely or above which relapse is probable. Schnittger *et al*[4] proposed a risk stratification score for *CBFB-MYH11*+ AML based on the quantification of fusion gene transcripts at diagnosis and after consolidation. So far, most MRD studies in *CBFB-MYH11*+ AML patients mainly used bone marrow (BM) samples. To evaluate the possibility to safely replace BM by peripheral blood (PB) for PCR-MRD monitoring in clinical settings, comparing RQ-PCR results of paired PB-BM samples is essential. For specific subtypes of AML, such as the t(8;21) positive AML with *AML1-ETO* transcripts, the possibility of MRD monitoring through quantitative analysis of PB has been reported.[5,6] Gallagher *et al*[7] reported on the prognostic value of RQ-PCR of *PML-RARA* transcripts and showed that paired PB-BM samples had

comparable MRD levels. They suggested that RQ-PCR for *PML-RARA* performed on PB at frequent follow-up points may improve predictive accuracy for relapse or continuing complete remission. Whether MRD monitoring of PB is of prognostic relevance in *CBFB-MYH11*+ AML patients is however still unknown.

PATIENTS AND METHODS

In this study, we quantified by RQ-PCR the *CBFB-MYH11* transcripts in 128 PB and 67 BM samples, including 64 paired PB-BM samples taken on the same day, from 10 different *CBFB-MYH11*+ AML patients. Patients' characteristics, type of breakpoint, cytogenetic data, treatment and clinical outcome are summarized in Table 1. RQ-PCR was performed as described before.[8] Results were expressed as normalized copy numbers (NCN): (numbers of *CBFB-MYH11* transcripts / numbers of β-glucuronidase (*GUS)* transcripts) x 10^4. *CBFB-MYH11* negative samples with less than 1000 transcripts of *GUS* were excluded from further analyses; this concerned 4 PB samples, two of which had a corresponding BM sample. At least 3 PB and 2 BM samples were analyzed per patient with a mean of 13 PB and 6 BM samples. The median follow-up was 27.5 months (range: 1.5 - 66 months). The number of samples and the timing of sampling was not uniform among patients due to the retrospective nature of our study.

RESULTS AND DISCUSSION

CBFB-MYH11 transcript levels in 8 diagnostic paired PB-BM samples showed no significant difference between both sample types (Figure 1). Out of 54 paired PB-BM samples obtained during follow-up, 16 pairs were MRD-negative in both BM and PB, while in 27 pairs MRD was detected in both BM and PB. In the remaining 11 paired follow-up samples MRD was detected only in BM, but not in the corresponding PB. MRD levels in the 54 follow-up PB-BM pairs showed a moderate correlation (Spearman correlation, r_s = 0.82): MRD levels in BM were generally higher than in PB, with a variable ratio between the MRD level in the BM sample and the corresponding PB sample, ranging from 0.8 up to almost 100 fold (Figure 1). This variable range implies that no straightforward relationship exists between MRD levels in paired PB and BM samples of *CBFB-MYH11*+ AML patients during follow-up. The generally higher MRD levels in BM are in line with the fact that AML has its origin in BM.

Although *CBFB-MYH11* transcript levels in follow-up PB did not precisely reflect MRD levels in the corresponding BM samples, monitoring of *CBFB-MYH11* transcripts in PB did have clinical and predictive value, as illustrated by the evolution and outcome of our 10 patients. Induction therapy resulted in a mean transcript reduction of 3.2 and 3.9 log in BM and PB respectively. The second cycle induced a mean tumor load reduction of 0.7 log in BM and 1.0 log in PB. After this second course, there were no molecular remissions in the BM samples. However, the molecular results of 8 patients with PB follow-up samples available after the second course, were as follows: 3 patients showed a molecular remission

Table 1. Clinical, molecular and karyotypic characteristics.

Patient	Sex	Age at diagnosis (years)	Type of transcript	Karyotype	Therapy	OS/DFS (months)	Clinical outcome
1	M	48	A	46,XY,inv(16)(p13q22)[10]	chemo [a], M-Allo	66 / 59	deceased in 2nd BM relapse
2	F	55	A	46,XX,inv(16)(p13q22)[11]	chemo, NM-Allo	48+ / 47+	1st CR
3	M	17	A	46,XY,inv(16)(p13q22)[10]	chemo, NM-Allo	45+ / 9	2nd CR after BM relapse
4	M	49	A	47,XY,+8,inv(16)(p13q22)[3] 47,XY,idem,del(7)(q32)[4] 47,XY, idem,-17,+22[2]	chemo	35+ / 34+	1st CR
5	F	51	A	46,XX,inv(16)(p13q22)[10]	chemo, NM-Allo	31+ / 10	2nd CR after BM relapse
6	M	11	A	46,XY,t(16;16)(p13;q22)[21]	chemo	1.5	deceased in 1st CR (treatment toxicity)
7	F	8	A	46,XX,inv(16)(p13q22)[7] 46,XX,add(7)(q22),inv(16)(p13q22)[4]	chemo, intrathecal chemo, Allo	24 / 18	deceased in 2nd CR after CNS relapse (transplant related)
8	M	67	D	46,XY,inv(16)(p13q22)[9] 47,XY,inv(16)(p13q22),+22[2]	chemo, Mylotarg [b], intrathecal chemo, cranial radiation	20+ / 13	2nd CR after BM and CNS relapse
9	F	68	A	46,XX,inv(16)(p13q22)[6] 47,XX,+8,inv(16)(p13q22)[8]	chemo, Zarnestra [c]	11+ / 10+	1st CR
10	F	31	A	ND	chemo	9+ / 8+	1st CR

[a] All patients were treated with standard induction regimens comprising combinations of idarubincine, Ara-C, mitoxantrone, etoposide, and amsacrine. After achieving complete remission, consolidation courses consisted of the same drugs used for induction. OS, overall survival; DFS, disease free survival; CR, complete remission; M-Allo, myelo-ablative allogeneic transplant; NM-Allo, non-myelo-ablative allogeneic transplant; ND, no data.

[b] 3 cycles of gemtuzumab ozogamicin (at relapse).

[c] farnesyl transferase inhibitor (post-consolidation).

and stayed in long term remission, while 5 patients had residual disease. In the 5 patients with MRD positivity in their PB after the second course, 4 relapses were seen (Figure 2): two patients suffered from an early BM relapse (patient 3 and 5), one patient had a combined BM-CNS relapse (patient 8), and one patient had a seemingly isolated CNS relapse (patient 7). In addition, a late BM relapse was

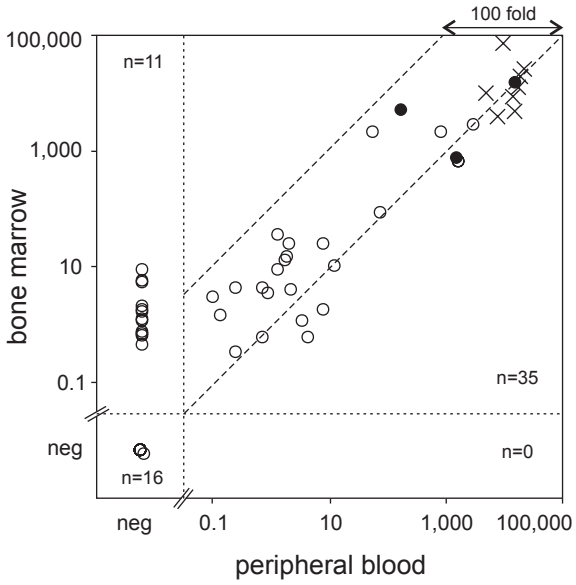


Figure 1.
Correlation of normalized copy numbers (NCN) x 10^4 between 62 paired PB and BM samples from 10 *CBFB-MYH11*+ AML patients. × represent paired diagnostic samples (n=8); ● represent paired cytological relapse samples (n=3); ○ represent paired follow-up samples in cytological remission (n=51); neg, MRD negative.

seen in patient 1 from who no molecular PB data were available after the second course (Figure 2). Thus, obtaining a molecular remission in PB after the second course may identify patients at low risk of relapse. In contrast to Schnittger *et al* [4] no correlation was found between the diagnostic BM or PB transcript levels and clinical outcome, nor between BM or PB tumor load reduction after induction or second course and clinical outcome, which might be due to the limited number of patients in our study.

In addition to providing prognostic information, MRD analysis may also be used for early detection of a (molecular) relapse. Indeed, all patients with a cytological BM relapse (patient 1, 3, 5, and 8), showed a significant (≥1 log) increase of *CBFB-MYH11* transcripts in their PB 2.5 to 4 months before cytological relapse (Figure 2). The patient who experienced an isolated CNS relapse (patient 7) after a molecular remission of 13 months in BM and PB, showed at the time of the CNS relapse low transcript levels in the BM; one month later, also the PB became positive (Figure 2).

In conclusion, our results show that there is no straight correlation between MRD levels in PB samples and the corresponding BM samples during follow-up in *CBFB-MYH11*+ AML. Nevertheless, monitoring of *CBFB-MYH11*

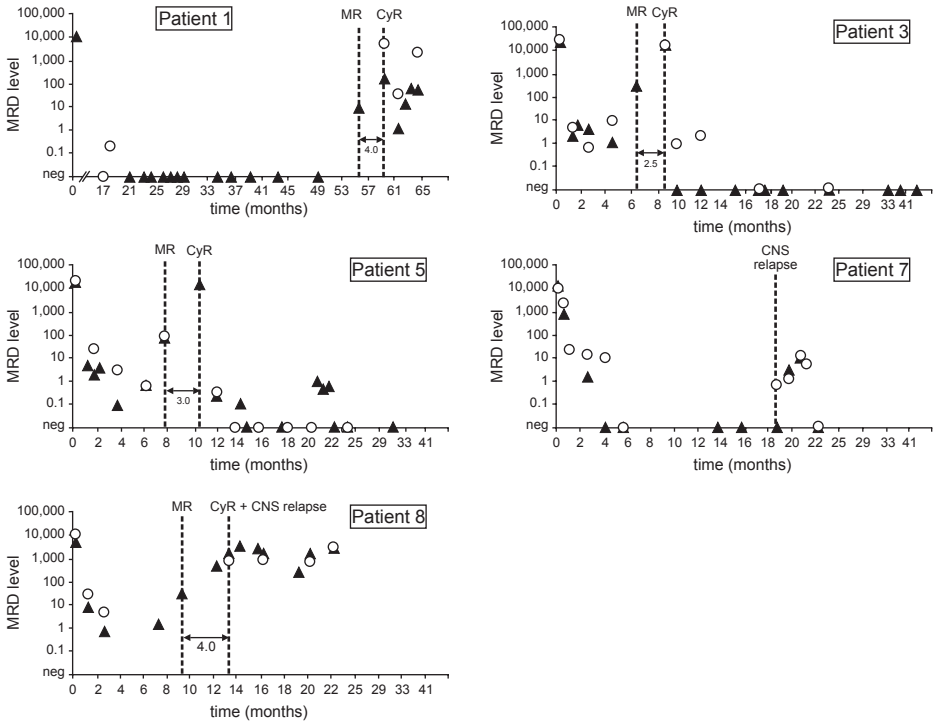


Figure 2. Evolution of MRD levels in 5 patients with a cytological BM and/or CNS relapse.
MRD levels are expressed as the ratio (*CBFB-MYH11* transcripts/*GUS* transcripts) x 10^4. ▲represent PB samples; ○ represent BM samples; MR, molecular relapse as defined by a ≥1 log increase of NCN; CyR, cytological BM relapse; the time between MR and CyR is indicated in months.

transcript levels in PB can have potential clinical value. Firstly, *CBFB-MYH11*-negative PB samples after the second course might predict a favorable prognosis with long term remission. Secondly, *CBFB-MYH11* levels in follow-up PB samples can predict disease reoccurrence 2.5 to 4 months prior to cytological BM relapse (Figure 2). Although analysis of BM could predict these relapses in some patients up to 2 months earlier, we believe that in practice a time frame of at least 2.5 months should be enough to adjust therapy in order to prevent the occurrence of a cytological relapse. Treatment intervention at the time of molecular relapse may result in better clinical outcome than treatment at the time of overt clinical relapse. This approach was proven to be successful for *PML-RARA*-positive patients.[9] Also for CML after allogeneic transplantation, it has been proven that patients benefit from early intervention with donor lymphocyte infusions at the time of cytogenetic relapse.[10] Based on our preliminary data, we propose that patients with a

CBFB-MYH11+ AML can be monitored through regular evaluation of their PB at set points, thereby avoiding multiple invasive BM punctures. Such PB examinations could be performed every 2 to 3 months after consolidation and BM should be examined in case of rising *CBFB-MYH11* transcripts in PB. However, further studies in a large population of uniformly treated patients with the collection of paired PB-BM samples at specific time-points are needed to determine the precise clinical value of PB samples for disease monitoring, as well as the optimal time interval between consecutive PB samples that allow a reliable early detection of molecular relapses in *CBFB-MYH11*+ AML. In addition, current therapeutic protocols should include treatment intervention at the time of molecular relapse, since this might improve patient outcome.

ACKNOWLEDGEMENTS

We are grateful to Prof. Dr. N. Blanckaert for his continuous support and to Marieke Comans-Bitter for preparation of the figures.

REFERENCES

1. Poirel H, Radford-Weiss I, Rack K, Troussard X, Veil A, Valensi F, Picard F, Guesnu M, Leboeuf D, Melle J. Detection of the chromosome 16 *CBF beta-MYH11* fusion transcript in myelomonocytic leukemias. *Blood* 1995; **85**: 1313-1322.
2. Mrozek K, Heinonen K, Bloomfield CD. Prognostic value of cytogenetic findings in adults with acute myeloid leukemia. *Int J Hematol* 2000; **72**: 261-271.
3. Buonamici S, Ottaviani E, Testoni N, Montefusco V, Visani G, Bonifazi F, Amabile M, Terragna C, Ruggeri D, Piccaluga PP, Isidori A, Malagola M, Baccarani M, Tura S, Martinelli G. Real-time quantitation of minimal residual disease in inv(16)-positive acute myeloid leukemia may indicate risk for clinical relapse and may identify patients in a curable state. *Blood* 2002; **99**: 443-449.
4. Schnittger S, Weisser M, Schoch C, Hiddemann W, Haferlach T, Kern W. New score predicting for prognosis in *PML-RARA*+, *AML1-ETO*+, or *CBFB-MYH11*+ acute myeloid leukemia based on quantification of fusion transcripts. *Blood* 2003; **102**: 2746-2755.
5. Tobal K, Newton J, Macheta M, Chang J, Morgenstern G, Evans PA, Morgan G, Lucas GS, Liu Yin JA. Molecular quantitation of minimal residual disease in acute myeloid leukemia with t(8;21) can identify patients in durable remission and predict clinical relapse. *Blood* 2000; **95**: 815-819.
6. Leroy H, Botton SD, Grardel-Duflos N, Darre S, Leleu X, Roumier C, Morschhauser F, Lai JL, Bauters F, Fenaux P, Preudhomme C. Prognostic value of real-time quantitative PCR (RQ-PCR) in AML with t(8;21). *Leukemia* 2005; **19**: 367-372.
7. Gallagher RE, Yeap BY, Bi W, Livak KJ, Beaubier N, Rao S, Bloomfield CD, Appelbaum FR, Tallman MS, Slack JL, Willman CL. Quantitative real-time RT-PCR analysis of *PML-RAR*alpha mRNA in acute promyelocytic leukemia: assessment of prognostic significance in adult patients from intergroup protocol 0129. *Blood* 2003; **101**: 2521-2528.

8. Gabert J, Beillard E, Van der Velden VHJ, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cave H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, Gonzalez M, Viehmann S, Malec M, Saglio G, Van Dongen JJM. Standardization and quality control studies of “real-time” quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) of fusion gene transcripts for minimal residual disease detection in leukemia - A Europe Against Cancer Program. *Leukemia* 2003; **12**: 2318-2357.
9. Lo-Coco F, Breccia M, Diverio D. The importance of molecular monitoring in acute promyelocytic leukaemia. *Best Pract Res Clin Haematol* 2003; **16**: 503-520.
10. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, Ljungman P, Ferrant A, Verdonck L, Niederwieser D, Van Rhee F, Mittermueller J, De Witte T, Holler E, Ansari H. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. *Blood* 1995; **86**: 2041-2050.

CHAPTER 3.4

Imatinib mesylate induces durable complete remission of advanced CML persisting after allogeneic bone marrow transplantation

Peter Vandenberghe[1,2], Nancy Boeckx[3], Elke Ronsyn[2], Ronny Decorte[4], Gregor Verhoef[5], Anne Hagemeijer[1]

[1]Department of Human Genetics, K.U. Leuven, Leuven, Belgium
[2]Laboratory of Experimental Hematology, K.U. Leuven, Leuven, Belgium
[3]Department of Laboratory Medicine, University Hospital Leuven, Leuven, Belgium
[4]Laboratorium voor Forensische Genetica en Moleculaire Archeologie, K.U. Leuven, Leuven, Belgium
[5]Department of Hematology, University Hospital Leuven, Leuven, Belgium

Adapted from letter. Leukemia 2003; 17: 458-460.

ABSTRACT

A 36-year old male underwent a non-related donor hematopoietic stem cell (HSC) transplantation after a non-myeloablative conditioning for chronic myeloid leukemia (CML) in second chronic phase. The early course after the transplantation was unfavorable, without sufficient engraftment of the allogeneic HSC and with the persistence of unstable advanced CML as indicated by a Ph-positive karyotype with clonal progression. In addition, there were contraindications against tapering of immunosuppression or therapeutic immunointervention with donor lymphocyte infusions. Four months after the HSC transplantation, treatment with imatinib mesylate was initiated. The administration of imatinib mesylate allowed a complete engraftment of donor HSC. Under continuing treatment, the patient reached a complete cytogenetic remission after 3 months, and molecular remission in peripheral blood as well as in bone marrow, in the absence of significant side effects. Our data indicate that imatinib mesylate induces durable complete remission of advanced CML persisting after allogeneic bone marrow transplantation.

INTRODUCTION

The development of imatinib mesylate (STI-571; Glivec; Gleevec; Novartis, Basel, Switzerland), a rationally designed inhibitor of the BCR-ABL protein tyrosine kinase, has marked the beginning of a new era in the treatment of CML. In first chronic phase, imatinib mesylate yields complete hematological remission rates of 98% and an unprecedented 60% rate of major or complete cytogenetic responses.[1] CML in myeloid blast crisis responds substantially less well with only 8% complete hematological and 7% complete cytogenetic responses, and a high frequency of early relapses.[2] As it stands today, allogeneic stem cell transplantation remains the only established curative treatment for CML, with a 10-year disease-free survival of approximately 50% for patients in early first chronic phase and with a matched sibling donor. Allotransplanted patients with a molecular, cytogenetic or hematological relapse can often be successfully rescued by discontinuation of immunosuppressive therapy and/or infusion of donor lymphocytes (DLI).[3] Little is known on the place of imatinib mesylate in the management of disease persistence or relapse after allogeneic stem cell transplantation for CML. Here we report a case of advanced CML that persisted after a failed unrelated bone marrow transplantation. Under treatment with imatinib mesylate, stable complete donor chimerism in the peripheral blood, improved graft function and a complete molecular response were achieved.

PATIENT, METHODS AND RESULTS

Chimerism studies

Short tandem repeats (STR) and the amelogenin locus were amplified using the AmpFLSTR SGM and Profiler Plus™ PCR Amplification Kits (Applied Biosystems, Lennik, Belgium), according to the manufacturer's instructions. The PCR products were analysed on the ABI 3100 genetic analyser. For informative STRs, the height of the donor-derived allele was divided by the sum of the heights of donor and receptor alleles. The percentage donor chimerism is the average of this value for all informative STRs, multiplied by 100.

Cytogenetics

Bone marrow specimens were cultured for cytogenetic analysis by established methods. Metaphases were R-banded. Chromosome aberrations are described according to guidelines of an International System for Human Cytogenetic Nomenclature.

Real-time quantitative polymerase chain reaction

RNA was isolated from peripheral blood or bone marrow mononuclear cells using the QIAamp Blood Kit (Qiagen, USA). cDNA synthesis (40 μl) was performed using 2 μg RNA, 200 units of M-MLV reverse transcriptase (Gibco BRL) and 25 μM random hexamers (Amersham Pharmacia Biotech, USA). The 25 μl RQ-PCR reaction mixture contained 5 μl of cDNA, TaqMan Universal PCR Master Mix 2x (Applied Biosystems), 300 nM primers, 200 nM TaqMan probes as described elsewhere.[4] The detection of the PCR products was based on TaqMan technology using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A standard curve was established using a serial dilution of *BCR-ABL* and β-glucuronidase plasmids (Ipsogen, Marseille, France). The copy numbers of the *BCR-ABL* fusion gene were normalized to the copy numbers of β-glucuronidase.

Clinical course and results

A 30-year old man was diagnosed with Ph-positive CML in chronic phase in December 1994. Under treatment with interferon-α-2b, a hematological response, but no cytogenetic response, was obtained. In early 2000, a myeloid blast crisis developed, and second chronic phase was reached only after intensive therapy with etoposide, topotecan and mitoxantrone. At this advanced disease stage, it was decided to perform an allogeneic bone marrow transplantation from an unrelated male donor with an immunogenic HLA-DRB1 subtype mismatch and an HLA-DQB1 mismatch (Recipient: DRB1*1302.DRB1*1501, DQB1*0602.DQB1*0604; donor: DRB1*1302.DRB1*1503, DQB1*0602.DQB1*0501). The patient now 36 years old, had poorly tolerated earlier intensive chemotherapy, therefore a non-myeloablative conditioning protocol was chosen, consisting of fludarabine, busulfan and antithymocyte globulin. Posttransplant immunosuppression included cyclosporin A and short course metotrexate. Initially, G-CSF supported hematological recovery was encouraging, but discontinuation of G-CSF led to a substantial decline of the leucocyte (Figure 1A) and platelet counts (Figure 1B). In addition, analysis of peripheral blood leukocyte chimerism over this time period revealed a concomitant decrease of donor chimerism from 25% (day 33) to less than 10% (day 84). Consecutive marrow examinations revealed hypocellular marrows, with a tendency to left shifting but not fulfilling cytological criteria for accelerated phase (Table 1). Karyotypes of these marrow aspirates continued to show almost uniform Ph positivity with additional clonal progression (46,XY,t(9;22)(q34;q11),add(17)(p11)), and with only a minority of (donor-derived) Ph-negative metaphases (Figure 1C). Meanwhile, the patient had cutaneous and hepatic acute GVHD grade II. Therefore, neither tapering of immunosuppression nor administration of DLI were considered reasonable salvage options.

Imatinib mesylate (CSTI-571-0114 protocol, Novartis) was started on day 124 after transplantation, at an initial dose of 400 mg/day and increased to 600 mg/day 2 weeks later. Within 6 weeks, a substantial improvement in the platelet count was observed, followed later by a more gradual increase of erythrocyte and leucocyte counts. Initially, this was accompanied by peripheral blood monocytosis and an increase in marrow monocytes and later, marrow blasts (Figure 1 A-B and Table 1). While these findings raised the suspicion of leukemia progression, a rapid increase of donor chimerism in the peripheral blood

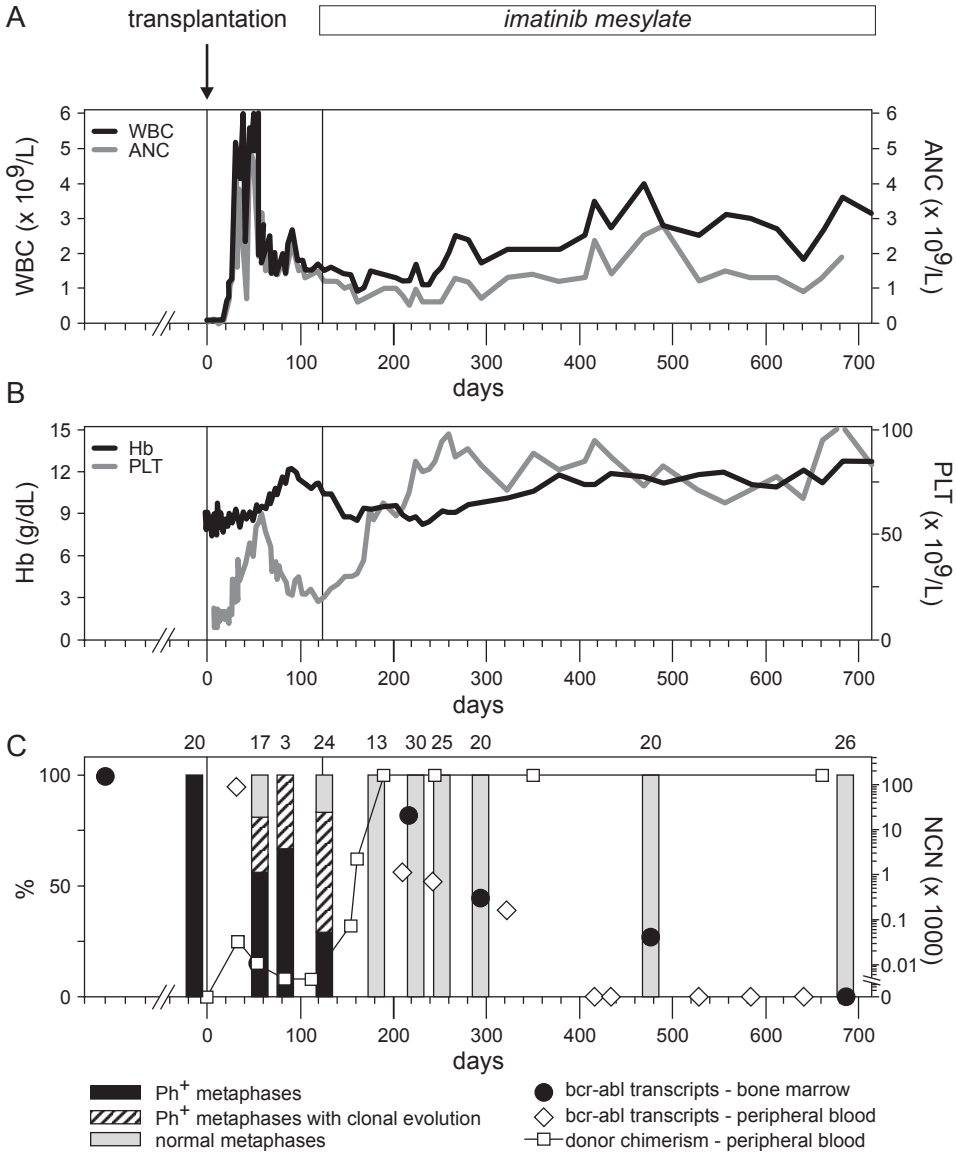

A
transplantation
imatinib mesylate
WBC
ANC
WBC (x 10^9/L)
ANC (x 10^9/L)
days
B
Hb
PLT
Hb (g/dL)
PLT (x 10^9/L)
days
C
20 17 3 24 13 30 25 20 20 26
%
NCN (x 1000)
days
Ph+ metaphases
Ph+ metaphases with clonal evolution
normal metaphases
bcr-abl transcripts - bone marrow
bcr-abl transcripts - peripheral blood
donor chimerism - peripheral blood

Figure 1. Evolution of peripheral blood counts, chimerism and disease burden after transplantation.
(A) Leucocyte count and absolute neutrophil count. The time course of leucocyte count (WBC, black line) and absolute neutrophil count (ANC, gray line) is shown.
(B) Hemoglobin level and platelet count. The time course of leucocyte count (Hb, black line) and platelet count (PLT, gray line) is shown.
(C) Molecular and cytogenetic analysis of engraftment and disease burden. *Peripheral blood leucocyte chimerism*: The evolution of donor chimerism (left Y-axis) is shown over time (solid line, white squares). *Bone marrow karyotype*: Bars show the distribution of R-banded normal metaphases (gray), Ph-positive metaphases (black) and Ph-positive metaphases with clonal evolution (stripes), as a function of time. The number of metaphases analysed is given above the bar. *BCR-ABL copy numbers in bone marrow and peripheral blood*: The copy numbers of the *BCR-ABL* fusion gene normalized to those of *β*-glucuronidase (NCN, right Y-axis) are shown as a function of time (solid dots represent bone marrow, open diamonds represent peripheral blood). The dot before transplantation represents a bone marrow sample 18 months before transplantation. In the RT-PCRs with negative results, mRNA was extracted from 3-9 x 10^6 cells. Drop lines represent day 0 (day of transplantation) and day 124 (start of imatinib mesylate).

was found reaching 100% between days 161 and 189 (days 37 and 65 of imatinib mesylate). This complete donor chimerism has since remained stable at 100% (last measured on day 661) (Figure 1C). In addition, complete cytogenetic remission was reached in the bone marrow within 8 weeks of imatinib mesylate. Finally, the number of *BCR-ABL* transcripts in peripheral blood, as measured by RQ-PCR, gradually declined over time, and since day 418 (day 294 of imatinib mesylate), the patient's peripheral blood has consistently tested negative on five consecutive occasions (Figure 1C). In addition, no *BCR-ABL* transcripts were detectable in a bone marrow aspirate on day 686 (day 562 of imatinib mesylate), indicating a disease burden of less than 1/100000 cells in the peripheral blood and bone marrow (Figure 1C). This molecular remission was confirmed with a nested RT-PCR (able to detect a 10^{-6} dilution of cDNA of a *BCR-ABL* positive cell line in control cDNA) on two samples from peripheral blood (days 584 and 641) as well as a bone marrow sample (day 686) (data not shown). Except for mild facial edema, there have been no significant imatinib mesylate-related toxicities. Cutaneous GVHD and disturbed liver tests attributed to hepatic GVHD have subsided allowing discontinuation of immunosuppression after 5 months of imatinib mesylate. Presently, the patient is free from complaints, and has resumed work, as well as strenuous physical activity. The increase of central blastosis and central and peripheral monocytosis that were seen after imatinib initiation has eventually resolved (Figure 1 A-B and Table 1).

DISCUSSION

At present only limited experience has been reported with imatinib mesylate in the treatment of CML after allogeneic transplantation. There are two cases of patients relapsing after allogeneic transplantation for CML and in whom imatinib mesylate treatment induced sustained hematological and cytogenetic remission.[5-6] Others have demonstrated that the association of imatinib mesylate and DLI can be highly effective as a treatment for Ph-positive acute leukemia relapsing after BMT.[7] Here, we report on the use of imatinib mesylate in a setting of persistent disease after allogeneic hematopoietic stem cell transplantation. There are several important observations.

Table 1. Leucocyte differential count in peripheral blood and bone marrow.

	Day -15	Day 84	Day 175	Day 217	Day 610	Day 686	Normal range
Peripheral blood							
Hgb (g/dl)	13.2	11.2	9.3	8.6	8.8	12.7	14-18
Platelets (x10^9/l)	346	28	62	70	85	102	150-450
Leucocytes (x10^9/l)	1.8	1.5	1.5	1.2	1.4	3.6	4-10
Leucocyte differential (%)							
Blasts		0	0	0	0	0	0
Promyelocytes		0	0	0	0	0	0
Myelocytes	1	2	0	0	1	0	0
Metamyelocytes	0	1	0	0	1	0	0
Banded granulocytes	0	11	0	0	0		0-5
Segmented granulocytes	76	72	58	59	45	52.9	35-79
Eosinophils	2	0	0	1	8	6.7	2.2-7.3
Basophils	0	0	0	0	0	0.3	1.5-3.5
Lymphocytes	13	4	19	23	32	31.2	20-50
Monocytes	8	0	23	17	13	8.9	2-10
Bone marrow							
Leucocyte differential (%)							
Blasts	1.5	1	3	7.5	7	3	0-5
Promyelocytes	3	6	4	10.5	5.5	7	4-10
Myelocytes	3	29	8.5	15	12	13.5	10-20
Metamyelocytes	1.5	3	8.5	2	1	14.5	15-25
Banded cells	1.5	10	8	13	7.5	16	10-25
Neutrophils	63	41	27.5	21	12.5	17.5	15-35
Eosinophils	1.5	1	1.5	6	12	1.5	2-5
Basophils	2.5	0	0	0	0	0	2-5
Lymphocytes	20.5	9	26	18	35.5	20	5-20
Monocytes	2	0	12	7	6.5	6	0-4

First, initiation of imatinib mesylate led to a rapid eradication of recipient hematopoiesis, as detected by chimerism analysis on peripheral blood. Second, despite the advanced disease stage, this was followed by a complete cytogenetic response achieved in the bone marrow within 2 months after initiation of therapy. Moreover, there was a gradual decrease of the number of *BCR-ABL* transcripts in peripheral blood, as well as in bone marrow, and a complete molecular remission (as verified with RQ-PCR and nested RT-PCR) was reached in peripheral blood as well as the bone marrow. This status of molecular remission in the peripheral blood has now been confirmed several times over a period spanning more than 250 days and it is hoped that it will be durable in the future. It is known that imatinib mesylate can induce cytogenetic remission in advanced CML. Yet, only a minority of patients with advanced CML enjoy complete cytogenetic responses or hematologic responses that are durable beyond 1 year. In addition, molecular responses to imatinib mesylate are rare in patients with chronic phase or more advanced CML.[1,2] Thus, while it cannot be excluded that the complete hematological, cytogenetic and molecular response in this patient was achieved by the sole action of imatinib mesylate, one could speculate that reduction of the disease burden by imatinib mesylate has created a window of opportunity for an alloreactive graft-versus-leukemia effect. Of note, imatinib mesylate has been reported to augment the function of antigen-presenting cells; as such it may also facilitate GVH-reactivity and GVL-effects.[8] Finally, despite low blood counts and peripheral blood donor chimerism at the start of imatinib, improved graft function was achieved without need for a second infusion of donor hematopoietic stem cells.

In summary, this case indicates that imatinib mesylate can be highly efficient to treat persisting CML after allogeneic hematopoietic stem cell transplantation. The observations also suggest that imatinib mesylate may promote or synergize with the graft-versus-leukemia effect after transplantation. More data are awaited to better define the role of imatinib mesylate after allogeneic stem cell transplantation.

ACKNOWLEDGEMENTS

PV is a Clinical Investigator of Fonds voor Wetenschappelijk Onderzoek Vlaanderen. ER is aspirant of Fonds voor Wetenschappelijk Onderzoek Vlaanderen. The assistance of the technical staff of the Leukemia Cytogenetics Laboratory, of Hilde Vranckx and Frans Houtmeyers is gratefully acknowledged. This text presents research results of the Belgian Programme of Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming. The scientific responsibility is assumed by the authors.

REFERENCES

1. Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, Niederwieser D, Resta D, Capdeville R, Zoellner U, Talpaz M, Druker B. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med* 2002; **346**: 645-652.
2. Sawyers CL, Hochhaus A, Feldman E, Goldman JM, Miller CB, Ottmann OG, Schiffer CA, Talpaz M, Guilhot F, Deininger MW, Fischer T, O'Brien SG, Stone RM, Gambacorti-Passerini CB, Russell NH, Reiffers JJ, Shea TC, Chapuis B, Coutre S, Tura S, Morra E, Larson RA, Saven A, Peschel C, Gratwohl A, Mandelli F, Ben-Am M, Gathmann I, Capdeville R, Paquette RL, Druker BJ. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* 2002; **99**: 3530-3539.
3. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, Ljungman P, Ferrant A, Verdonck L, Niederwieser D. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. *Blood* 1995; **86**: 2041-2050.
4. Stentoft J, Pallisgaard N, Kjeldsen E, Holm MS, Nielsen JL, Hokland P. Kinetics of *BCR-ABL* fusion transcript levels in chronic myeloid leukemia patients treated with STI571 measured by quantitative real-time polymerase chain reaction. *Eur J Haematol* 2001; **67**: 302-308.
5. Olavarria E, Craddock C, Dazzi F, Marin D, Marktel S, Apperley JF, Goldman JM. Imatinib mesylate (STI571) in the treatment of relapse of chronic myeloid leukemia after allogeneic stem cell transplantation. *Blood* 2002; **99**: 3861-3862.
6. Wassmann B, Klein SA, Scheuring U, Pfeifer H, Martin H, Gschaidmeier H, Hoelzer D, Ottmann OG. Hematologic and cytogenetic remission by STI571 (Glivec) in a patient relapsing with accelerated phase CML after second allogeneic stem cell transplantation. *Bone Marrow Transplant* 2001; **28**: 721-724.
7. Baron F, Frere P, Fillet G, Beguin Y. Treatment of leukemia relapse after allogeneic hematopoietic stem cell transplantation by donor lymphocyte infusion and STI-571. *Haematologica* 2001; **86**: 993-994.
8. Wang HW, Cheng FD, Cuenca AG, Bhalla K, Sotomayor EM. STI-571, a Selective Tyrosine Kinase Inhibitor, Significantly Enhances Antigen Presentation by Bone Marrow Derived APCs: Implications for Immunotherapy of Tumors-Derived from Antigen Presenting Cells. *Blood* 2001; **98**: 16a.

CHAPTER 3.5

High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg®) treatment in acute myeloid leukemia patients

Vincent H.J. van der Velden[1], Nancy Boeckx[2], Inge Jedema[3], Jeroen G. te Marvelde[1], Patricia G. Hoogeveen[1], Marc Boogaerts[4], Jacques J.M. van Dongen[1]

[1]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
[2]Department of Laboratory Medicine, Laboratory of Hematology, University Hospital Gasthuisberg, Leuven, Belgium
[3]Department of Hematology, LUMC, Leiden, The Netherlands
[4]Department of Hematology, University Hospital Gasthuisberg, Leuven, Belgium

Published in Leukemia 2004; 18: 983-988.

ABSTRACT

Gemtuzumab ozogamicin (Mylotarg®) induces remission in approximately 30% of relapsed AML patients. We previously demonstrated that gemtuzumab infusion results in near-complete CD33 saturation in peripheral blood, and that saturating gemtuzumab levels result in continuous binding and internalization of gemtuzumab due to renewed CD33 expression. We now demonstrate that a high CD33-antigen load in peripheral blood is an independent adverse prognostic factor, likely due to peripheral consumption of gemtuzumab. Indeed, CD33 saturation in bone marrow is significantly reduced (40-90% saturation) as compared with CD33 saturation in corresponding peripheral blood samples (>90%). *In vitro*, such reduced CD33 saturation levels were strongly related with reduced cell kill. Apparently, high CD33-antigen loads in blood consume gemtuzumab and thereby limit its penetration into bone marrow. Consequently CD33 saturation in bone marrow is reduced, which hampers efficient cell kill. Therefore, gemtuzumab should be administered at higher or repeated doses, or, preferably, after reduction of the leukemic cell burden by classical chemotherapy.

INTRODUCTION

Gemtuzumab ozogamicin (Mylotarg®) is a humanized CD33 antibody linked to the anti-tumor antibiotic calicheamicin.[1] In phase-II clinical trials, gemtuzumab administered as single agent at two doses of 9 mg/m^2 two weeks apart, resulted in complete remission in approximately 30% of relapsed AML patients.[2, 3] Gemtuzumab therapy was related with low toxicity, the major complication being occurrence of hepatic veno-occlusive disease (VOD) in a small number of patients.[4] Based on these results, gemtuzumab was FDA-approved for treatment of relapsed AML patients over 60 years of age.[5]

We have previously shown that after intravenous infusion of gemtuzumab, near-complete saturation (>90%) of CD33-antigens is achieved on AML blast cells present in peripheral blood (PB).[6] In addition, gemtuzumab also binds to CD33-antigens expressed by monocytes and granulocytes present in PB at the time of infusion.[6] Consequently, the total amount of gemtuzumab bound to CD33-antigens in PB depends on the number of CD33-positive monocytes, granulocytes, and AML blast cells as well on the CD33 expression level of these cells (referred to as the peripheral CD33-antigen load). After binding of gemtuzumab to CD33-antigens, the CD33-antigen/gemtuzumab complexes are rapidly internalized,[6] followed by hydrolysis of the acid-labile linker and subsequent activation of the calicheamicin γ_1 derivate.[1] Of importance, upon internalization of the complexes, new CD33-antigens are rapidly expressed on the cell surface and subsequently can bind gemtuzumab. The rate of renewed CD33-antigen expression is related to the degree of CD33 saturation and

internalization[6] and to the activation status of the cell.[7] Consequently, the process of CD33 saturation, internalization, and new CD33-antigen expression is ongoing as long as saturating gemtuzumab concentrations are maintained. This continuous process will result in a progressive intracellular accumulation of gemtuzumab.

Obviously, high levels of intracellularly accumulated calicheamicin are more toxic than low levels. Indeed, patients with less than 5% AML blasts in their PB after the first gemtuzumab infusion had higher maximal gemtuzumab binding levels on AML blast cells (i.e. stronger CD33-antigen expression) than patients with more than 5% AML blasts in PB after the first infusion.[6] However, no relation was found between CD33 expression levels and clinical outcome as assessed by analysis of bone marrow (BM) samples.[2, 3] Apparently, the gemtuzumab response in PB, but not in BM, is related to the CD33 expression level on AML blasts.

We hypothesized that high CD33-antigen loads in peripheral blood consume intravenously administered gemtuzumab, thereby limiting gemtuzumab concentrations in BM. As a consequence, CD33 saturation of AML blasts in BM might be reduced, resulting in greatly reduced intracellular accumulation of gemtuzumab and thereby in inefficient cell kill.

MATERIALS AND METHODS

Patients

Patients enrolled in the gemtuzumab phase-II clinical protocols 0903B1-202-EU (n=54) and 0903B1-203-EU (n=38) were studied.[2, 3, 6] In these open, single-arm multi-center phase II studies, gemtuzumab (Wyeth-Ayerst Laboratories, St. Davids, PA, USA) was administered as a single 2-hour intravenous infusion at a dose of 9 mg/m^2. In general, each patient received two gemtuzumab doses with at least 14 days between the doses. Prior to the start of each gemtuzumab treatment cycle and 3 and 6 hours after the start of each gemtuzumab treatment cycle, PB samples were obtained, immediately placed on ice (4°C) and shipped overnight at 4°C to Immunology, Erasmus MC, Rotterdam by express courier (World Courier, Hoofddorp, The Netherlands).

Six patients treated with gemtuzumab on compassionate use basis were also included in this study. BM samples were taken prior to and 24 h after gemtuzumab infusion. The latter sample was immediately diluted in 200-ml ice-cold phosphate-buffered saline and kept on ice until further analysis to limit additional CD33 saturation due to the presence of gemtuzumab in contaminating PB. PB samples were taken just prior to gemtuzumab infusion and 6 and 24 h thereafter. Inclusion of additional patients treated with gemtuzumab as a single agent and at a dose of 9 mg/m^2 was not possible, because current European clinical trails employ another gemtuzumab dose and/or use a combination regimen with chemotherapy.

Analysis of CD33 expression, CD33 saturation, and maximal gemtuzumab binding

Analysis of CD33 expression, CD33 saturation, and maximal gemtuzumab binding was performed as described previously using a flow cytometric assay. [6] Briefly, to determine CD33 saturation cells were incubated with biotin-conjugated mouse anti-human IgG_4 antibodies (Caltag Laboratories, Burlingame, CA, USA), followed by streptavidin-FITC (Biosource, Nivelles, Belgium) as a second step reagent. To detect maximal gemtuzumab binding, cells were first incubated with excess gemtuzumab (final concentration: 10 µg/ml), followed by successive incubation with biotin-conjugated mouse anti-human IgG_4 antibodies and streptavidin-FITC. As a negative control, cells were incubated with streptavidin-

FITC alone. To detect CD33 antigenic sites not occupied by gemtuzumab, cells were incubated with CD33-PE (clone P67.6; BD Biosciences, San Jose, CA, USA) or, as a negative control, IgG_1-PE (BD Biosciences). All incubations were performed at 4°C. CD45-PerCP (2D1; BD Biosciences) and/or CD14-PE (My4; Coulter Clone, Hialeah, FL, USA) antibodies were included to distinguish between various cell populations.

To determine the relation between gemtuzumab concentration and CD33 saturation, the AML193 cell line or PB from healthy volunteers was incubated with various gemtuzumab concentrations (15 minutes at 4°C), after which CD33 saturation was analyzed.

Analysis of peripheral CD33-antigen load

Samples taken just prior to gemtuzumab infusion were subjected to routine morphological analysis to assess the leukocyte differential. In addition, maximal gemtuzumab binding to AML blast cells, monocytes and granulocytes was determined as described above. The peripheral CD33-antigen load was subsequently calculated as follows: (maximal gemtuzumab binding blasts x #blasts/L) + (maximal gemtuzumab binding monocytes x #monocytes/L) + (maximal gemtuzumab binding granulocytes x #granulocytes/L).

Analysis of cell kill

To determine the relationship between induction of cell kill and gemtuzumab concentration, AML193 cells were incubated with various gemtuzumab concentrations for 24 h or 40 h, after which cell kill was determined by ^{51}Cr release assay as described previously.[7, 8] Because AML193 cells also can take up gemtuzumab via CD33-independent mechanisms (which will contribute to its final cytotoxic activity),[7] an isotype-matched calicheamicin-conjugated antibody was not included as a control. To determine whether free calicheamicin (e.g. due to hydrolysis of the linkers during the incubation period) could contribute to cell kill, pilot experiments using free calicheamicin were performed. These experiments showed that the maximal amount of free calicheamicin that could be present during the incubation period (<0.015 µg/ml at the highest gemtuzumab dose; based on less than 1% free drug in clinical grade gemtuzumab and an estimated increase with maximally 2-3% in 24 hours; Philip Hamann, personal communication) resulted in <15% cell kill in our assay.[7]

Statistical analysis

To determine whether the peripheral CD33-antigen load was different between patients that achieved complete remission (either with or without full recovery of platelets[2, 3]) and non-responders, a Mann-Whitney *U* test using log-transformed data was applied. Univariate analysis using age, FAB-type, multidrug resistance efflux, ECOG performance status, duration of CR1, and percentage BM blasts[3] was performed using the Mann-Whitney *U* test. Karyotype data were unfortunately not completely available; inclusion of fusion gene transcript data (*CBFB-MYH11*, *PML-RARA*, *AML1-ETO*) was not done due to the very low number of patients (3 out of 92) being positive for one of these translocation-associated fusion gene transcripts. Multivariate analysis, including all indicated variables that were significant in the univariate analysis, was performed by logistic regression. In all tests, a *p* value of less then 0.05 was considered significant.

RESULTS AND DISCUSSION

High peripheral CD33-antigen load is an independent prognostic factor

To evaluate whether a high peripheral CD33-antigen load was related to clinical outcome, we analyzed the CD33-antigen load in PB of 92 patients enrolled in the European phase-II protocols. The mean peripheral CD33-antigen load was significantly lower in patients who achieved complete remission than in non-

responders (mean log(peripheral CD33-antigen load)±SD: 3.67±0.85 versus 4.58±0.97; Table 1). Further statistical analysis using age, FAB-type, multidrug resistance efflux, ECOG performance status, duration of CR1, and percentage BM blasts [3] showed that duration of CR1 and percentage BM blasts were significant prognostic markers as well (Table 1). However, multivariate analysis showed that only the log(peripheral CD33-antigen load) was an independent prognostic factor (Table 1). In accordance with our data, the likelihood of achieving response to gemtuzumab therapy was shown to be associated with lower levels of CD33-positive leukemic blasts in PB [3] or BM. [2] Thus, high peripheral CD33-antigen loads reduce treatment efficacy, probably by consuming gemtuzumab and thereby reducing the gemtuzumab amount available for leukemic cells in BM. This may result in reduced CD33 saturation on AML blast cells present in BM and subsequently in inefficient induction of cell kill.

Table 1. Results of statistical analysis for prognostic factors.

Analysis	*Prognostic variable*[a]	*Univariate analysis*[b]	*Multivariate analysis*[c]
CR(p) *vs* NR[d]	Age	0.566	n.d.
	FAB	0.508	n.d.
	Percentage blasts in BM	**0.046**	0.272
	Multidrug resistance efflux	0.888	n.d.
	ECOG performance status	0.600	n.d.
	Duration of CR1	**0.009**	0.201
	CD33-antigen load PB	**0.001**	**0.014**

[a] For details see reference.[3]
[b] Mann-Whitney *U* test.
[c] Logistic regression.
[d] CR(p): patients in complete remission, with or without full recovery of platelets (n=18); NR: non-responders (n=74).

CD33 saturation on AML blast cells present in BM is reduced

To investigate whether peripheral gemtuzumab consumption indeed could reduce the gemtuzumab amount available for leukemic cells in BM, we analyzed BM samples obtained 24 h after gemtuzumab infusion. As shown in Figure 1A, prior to gemtuzumab infusion only background saturation levels (<20%) were observed in PB and BM. In PB, near complete CD33 saturation was observed at 6 h (data not shown) and at 24 h after gemtuzumab infusion (Figure 1A). In contrast, CD33 saturation in BM was significantly lower and ranged from 42% to 90%. Apparently, penetration of gemtuzumab (given at a dose of 9 mg/m^2) into BM is not sufficient to completely saturate CD33-antigens on AML blasts present in BM 24 h after gemtuzumab infusion.

Preliminary analysis showed that the degree of reduced CD33 saturation in BM was only partly related to the peripheral CD33-antigen load (data not shown), but

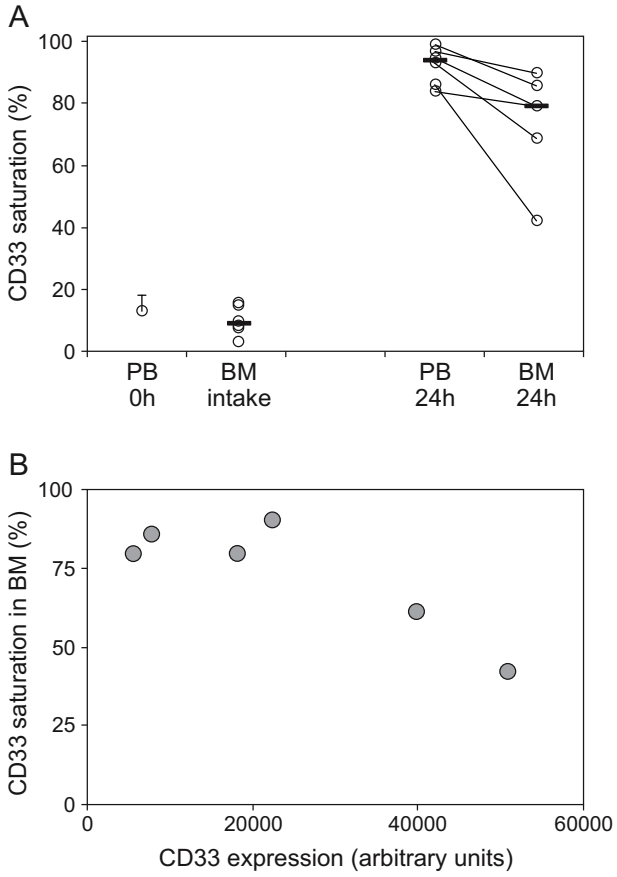


Figure 1. CD33 saturation levels in BM.

A. CD33 saturation on AML blast cells in BM and PB samples obtained prior to and 24 h after gemtuzumab infusion in six AML patients. Before gemtuzumab infusion only background CD33 saturation levels (<20%) were observed in BM. For comparison, previously published CD33 saturation levels in PB prior to gemtuzumab infusion (mean ± SD) are shown as well. [6] Twenty-four hours after gemtuzumab infusion, CD33 saturation levels in PB were near-complete (mean: 92%), whereas CD33 saturation levels in BM were significantly lower (mean: 74%; $p<0.05$ by Mann-Whitney test). The horizontal bars indicate the mean CD33 saturation.

B. Relation between CD33 saturation by AML blast cells in BM and CD33 expression by blast cells for the same patients. High CD33 expression levels were related to lower CD33 saturation, suggesting that also the CD33-antigen load in BM contributes to CD33 saturation in BM (Pearson Correlation: -0.867; $p<0.05$). The six patients analyzed in our study all died: five due to disease progression and one due to stroke. Two patients (with 61% and 80% CD33 saturation in bone marrow) showed an initial response after gemtuzumab infusion, characterized by a strong reduction in blast cells in peripheral blood (to levels <2%). Both patients however showed a fast increase in blast cells in peripheral blood 1-2 weeks later. It can be speculated that this is due to outgrowth of AML blast cells in BM, which were not sufficiently saturated with gemtuzumab for efficient induction of cell kill.

was related to the CD33 expression level of the AML blasts in BM (Figure 1B). It will be interesting to analyze the relationship between peripheral CD33-antigen loads and CD33 saturation in BM in future protocols involving gemtuzumab in more detail. However, it should be kept in mind that the final CD33 saturation level in BM is not only affected by the peripheral CD33-antigen load, but may also be dependent on the total CD33-antigen load in BM.

Complete saturation is required for efficient cell kill

After binding to CD33-antigens, gemtuzumab is rapidly internalized and the calicheamicin derivate is intracellularly activated.[1,6] Importantly, continuous renewed expression of CD33 antigens on the cellular surface of myeloid cells significantly increases the amount of internalized gemtuzumab as long as saturating levels of gemtuzumab are present.[6] Therefore, the reduced CD33 saturation levels observed in BM may result in severely reduced accumulation of gemtuzumab into the cell, and thereby in inefficient induction of cell kill. To evaluate whether reduced CD33 saturation levels indeed resulted in less efficient induction of cell kill, myeloid cells (monocytes, granulocytes, AML193 cells) were incubated with gemtuzumab, and CD33 saturation and/or cell kill were determined. As shown in Figure 2, CD33 saturation and gemtuzumab-induced cell kill were both concentration dependent. Of importance, the combined CD33 saturation and cell kill data indicate that CD33 saturation levels <90% (as found in BM) resulted in a dramatic decrease in cell lysis (<5%; Figure 2C). Thus, gemtuzumab concentrations resulting in near complete CD33 saturation levels (>90%) are required for efficient cell kill. In our opinion this reflects the continuous process of saturation, internalization, and new expression of CD33-antigens,[6,9] resulting in the progressive accumulation of gemtuzumab (and consequently calicheamicin) in the cell (see Figure 3A). In agreement with this, the steep increase in gemtuzumab-induced cell kill (from 15% to >45%) is observed in the gemtuzumab concentration range of 0.5 to 2.5 µg/ml; at these concentrations the vast majority of the gemtuzumab-induced cell kill is CD33-mediated.[7] Additionally, at such high gemtuzumab concentrations (>1 µg/ml) CD33-independent uptake mechanisms (i.e. endocytosis) may occur.[7] Irrespective whether gemtuzumab is taken up by the cell via CD33-dependent internalization or via CD33-independent mechanisms, our data indicate that high gemtuzumab concentrations (those resulting in near complete CD33 saturation) are needed for efficient cell kill. The reduced CD33 saturation levels in BM (Figure 1) indicate that the gemtuzumab concentration in BM is relatively low, and consequently cell kill will be reduced (Figure 2).

Implications for gemtuzumab therapy

In this report we show that a high peripheral CD33-antigen load is an independent adverse prognostic factor in AML patients treated with gemtuzumab. Our data indicate that a high peripheral CD33-antigen load consumes a large part of the

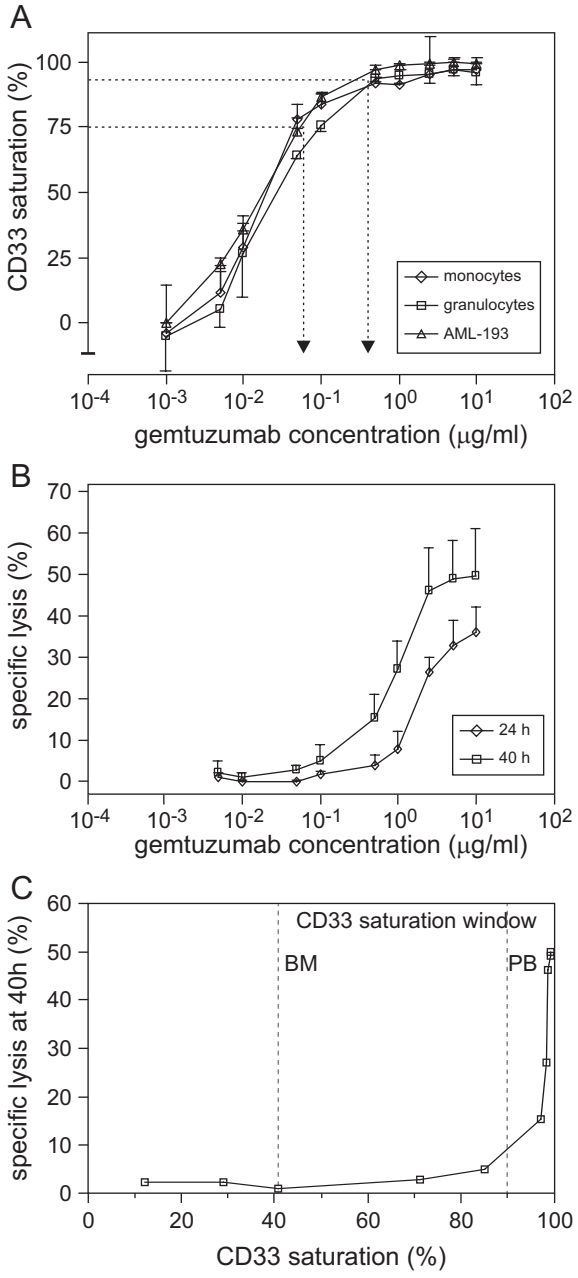

A
CD33 saturation (%)
100
75
50
25
0
monocytes
granulocytes
AML-193
10-4
10-3
10-2
10-1
100
101
102
gemtuzumab concentration (μg/ml)
B
specific lysis (%)
70
60
50
40
30
20
10
0
24 h
40 h
gemtuzumab concentration (μg/ml)
C
specific lysis at 40h (%)
CD33 saturation window
BM
PB
0
20
40
60
80
100
CD33 saturation (%)

Figure 2. Relation between CD33 saturation and gemtuzumab-induced cell kill.
A. CD33 saturation of AML193 cells in relation to gemtuzumab concentration (mean ± SD; n=3). For CD33 saturation levels >90% (as observed in PB), a gemtuzumab concentration of at least 0.2 µg/ml was needed. For CD33 saturation levels of 40-90% (as observed in BM), gemtuzumab concentrations of 0.01-0.1 µg/ml (i.e. 2 to 20-fold lower) were needed. The dotted lines indicate the mean CD33 saturation level in BM (74%) or PB (92%) and the corresponding gemtuzumab concentration. Comparable data were obtained for granulocytes ($CD33^{+(low)}$; n=3) and monocytes ($CD33^{+(high)}$; n=3).
B. Lysis of AML193 cells in relation to gemtuzumab concentration (mean ± SD; n=3). Lysis was determined using a ^{51}Cr release assay after incubation with various gemtuzumab concentrations for 24 h or 40 h.[7, 8] The steep increase in cell kill observed at gemtuzumab concentration ranging from 0.1 to 2.5 µg/ml (from <5% to >45% cell kill) is assumed to be gemtuzumab-specific, because the vast majority of cell kill can be inhibited by addition of an excess of CD33-antibody.[7] At higher gemtuzumab concentrations, also CD33-independent uptake of gemtuzumab may be involved.[7]
C. Relation between CD33 saturation and cell lysis of AML193 cells. The combined data of **A** and **B** show that CD33 saturation levels >90% (as observed in PB; CD33 saturation window PB) resulted in 10-50% lysis. CD33 saturation levels <90% as observed in BM (CD33 saturation window BM) result in <5% lysis.

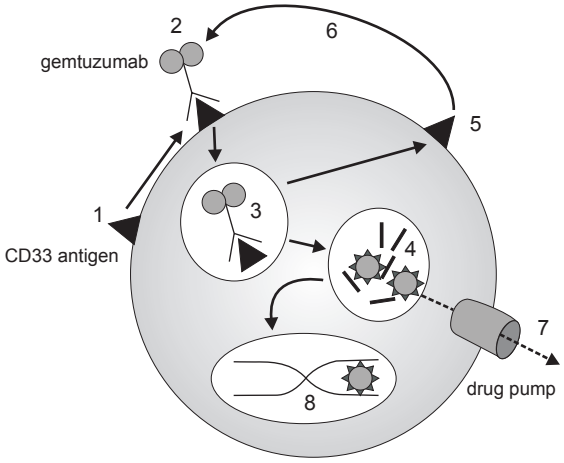
gemtuzumab dose (9 mg/m^2), which results in reduced gemtuzumab penetration in BM (Figure 3B). As a consequence, CD33 saturation of AML blast cells present in BM is incomplete, which drastically hampers subsequent cell kill.

For efficient killing of AML blasts in BM, the effective gemtuzumab dose should be increased in order to reach consistently high CD33 saturation levels. This can be done by increasing the dose of 9 mg/m^2 or by giving repeated doses at subsequent days,[9] which may however be complicated by the increase of severe side effects, including VOD.[4] Alternatively and preferably, gemtuzumab could be administered *after* reduction of the leukemic cell burden by classical chemotherapy. However, so far, in most studies applying gemtuzumab-based combination regimens, gemtuzumab is given prior to or simultaneously with chemotherapy.[10-15] The use of gemtuzumab in a post-remission regimen, combined with chemotherapy, has recently shown to be feasible and well-tolerated but needs further investigation.[16] To further improve the intracellular accumulation of gemtuzumab, drug efflux modulators such as cyclosporine A may be added to the treatment regimen as well.[13-18] A better understanding of gemtuzumab's mechanism of action will hopefully contribute to the design of future treatment protocols which maximally exploit the potencies of gemtuzumab.

ACKNOWLEDGEMENTS

We thank Marieke Comans-Bitter for preparation of the figures, Mark Berger, Mat Sherman, Cathy Eten, Gerry Messerschmidt and Chantal Lejeune for support with the gemtuzumab protocols 0903B1-202-EU and 0903B1-203-EU, Philipp Hamann for helpful discussions, Dr. W.C.J. Hop for help with statistical analyses, and Dr. Ton Langerak, Dr. Mirjam van der Burg, and Dr. Jon Laman for critically reading the manuscript.

A. Cellular parameters affecting the efficacy of gentuzumab



1. CD33 expression level
2. CD33 saturation
3. Internalization
4. Activation of calicheamicin
5. New expression of CD33
6. Binding of gemtuzumab
7. Efflux of calicheamicin
8. Induction of DNA breaks

B. Systemic parameters affecting the efficacy of gentuzumab

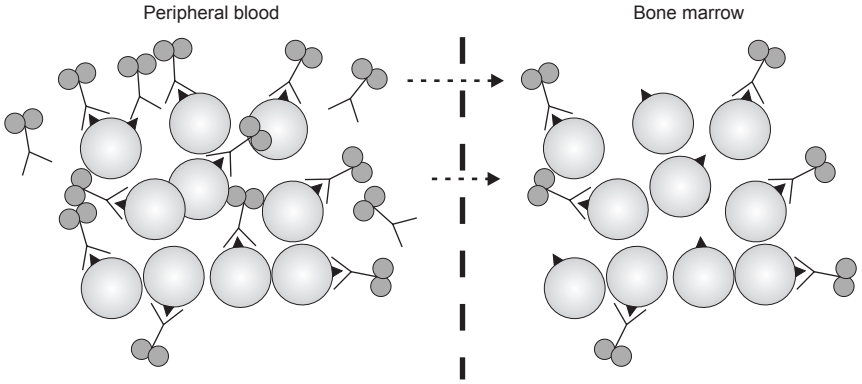


Figure 3. Parameters affecting the efficacy of gemtuzumab treatment.
A. At the cellular level, several parameters affecting gemtuzumab's efficacy can be recognized. The maximal gemtuzumab binding is dependent on the CD33 expression level of the cell (1). After gemtuzumab infusion, CD33-antigens are rapidly saturated (2) and the gemtuzumab/CD33-antigen complexes are subsequently internalized (3). In lysosomes, the acid-labile linker is hydrolyzed, followed by activation of calicheamicin and degradation of the antibody part of gemtuzumab (4). Upon internalization, new CD33-antigens are rapidly expressed on the cell membrane (5) and can bind gemtuzumab (6), resulting in an ongoing process of gemtuzumab internalization as long as saturating gemtuzumab concentrations are present. Calicheamicin can be pumped out of the cell by drug pumps such as Pgp and MRP1 (7). Active calicheamicin enters the nucleus and can induce double-strand DNA breaks (8), finally resulting in the induction of cell death.
B. Besides cellular parameters, systemic parameters are involved in the efficacy of gemtuzumab. These particularly include the peripheral CD33-antigen load, which determines the amount of gemtuzumab that is consumed by the myeloid cells in PB and consequently the amount of gemtuzumab available for binding to CD33-antigens in BM. CD33 saturation in BM also appeared to be affected by the total CD33-antigen load in BM.

REFERENCES

1. Hamann PR, Hinman LM, Hollander I, Beyer CF, Lindh D, Holcomb R, Hallett W, Tsou HR, Upeslacis J, Shochat D, Mountain A, Flowers DA, Bernstein I. Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug Chem* 2002; **13**: 47-58.
2. Larson RA, Boogaerts M, Estey E, Karanes C, Stadtmauer EA, Sievers EL, Mineur P, Bennett JM, Berger MS, Eten CB, Munteanu M, Loken MR, Van Dongen JJM, Bernstein ID, Appelbaum FR. Antibody-targeted chemotherapy of older patients with acute myeloid leukemia in first relapse using Mylotarg (gemtuzumab ozogamicin). *Leukemia* 2002; **16**: 1627-1636.
3. Sievers EL, Larson RA, Stadtmauer EA, Estey E, Löwenberg B, Dombret H, Karanes C, Theobald M, Bennett JM, Sherman ML, Berger MS, Eten CB, Loken MR, Van Dongen JJM, Bernstein ID, Appelbaum FR. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J Clin Oncol* 2001; **19**: 3244-3254.
4. Giles FJ, Kantarjian HM, Kornblau SM, Thomas DA, Garcia-Manero G, Waddelow TA, David CL, Phan AT, Colburn DE, Rashid A, Estey EH. Mylotarg (gemtuzumab ozogamicin) therapy is associated with hepatic venoocclusive disease in patients who have not received stem cell transplantation. *Cancer* 2001; **92**: 406-413.
5. Bross PF, Beitz J, Chen G, Chen XH, Duffy E, Kieffer L, Roy S, Sridhara R, Rahman A, Williams G, Pazdur R. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clin Cancer Res* 2001; **7**: 1490-1496.
6. Van der Velden VHJ, Te Marvelde JG, Hoogeveen PG, Bernstein ID, Houtsmuller AB, Berger MS, Van Dongen JJM. Targeting of the CD33-calicheamicin immunoconjugate Mylotarg (CMA-676) in acute myeloid leukemia: *in vivo* and *in vitro* saturation and internalization by leukemic and normal myeloid cells. *Blood* 2001; **97**: 3197-3204.
7. Jedema I, Barge RMY, Van der Velden VHJ, Nijmeijer BA, Te Marvelde JG, Van Dongen JJM, Willemze R, Falkenburg JHF. Internalization and cell cycle dependent killing of leukemic cells by gemtuzumab ozogamicin (GO): rationale for application in CD33 negative malignancies with endocytic capacity. *Leukemia* 2004; **18**: 316-325.

8. Jedema I, Barge RM, Willemze R, Falkenburg JH. High susceptibility of human leukemic cells to Fas-induced apoptosis is restricted to G(1) phase of the cell cycle and can be increased by interferon treatment. *Leukemia* 2003; **17**: 576-584.
9. Van der Velden VHJ, Berger MS, Van Dongen JJM. Mylotarg therapy in acute myeloid leukemia: Mechanism of action and implications for future treatment protocols. *Haematology and Blood Transfusion* 2003; **41**: 169-176.
10. Giles F, Estey E, O'Brien S. Gemtuzumab ozogamicin in the treatment of acute myeloid leukemia. *Cancer* 2003; **98**: 2095-2104.
11. Alvarado Y, Tsimberidou A, Kantarjian H, Cortes J, Garcia-Manero G, Faderl S, Thomas D, Estey E, Giles FJ. Pilot study of Mylotarg, idarubicin and cytarabine combination regimen in patients with primary resistant or relapsed acute myeloid leukemia. *Cancer Chemother Pharmacol* 2003; **51**: 87-90.
12. Kell WJ, Burnett AK, Chopra R, Yin JA, Clark RE, Rohatiner A, Culligan D, Hunter A, Prentice AG, Milligan DW. A feasibility study of simultaneous administration of gemtuzumab ozogamicin with intensive chemotherapy in induction and consolidation in younger patients with acute myeloid leukemia. *Blood* 2003; **102**: 4277-4283.
13. Tsimberidou A, Cortes J, Thomas D, Garcia-Manero G, Verstovsek S, Faderl S, Albitar M, Kantarjian H, Estey E, Giles FJ. Gemtuzumab ozogamicin, fludarabine, cytarabine and cyclosporine combination regimen in patients with CD33+ primary resistant or relapsed acute myeloid leukemia. *Leuk Res* 2003; **27**: 893-897.
14. Tsimberidou A, Estey E, Cortes J, Thomas D, Faderl S, Verstovsek S, Garcia-Manero G, Keating M, Albitar M, O'Brien S, Kantarjian H, Giles F. Gemtuzumab, fludarabine, cytarabine, and cyclosporine in patients with newly diagnosed acute myelogenous leukemia or high-risk myelodysplastic syndromes. *Cancer* 2003; **97**: 1481-1487.
15. Apostolidou E, Cortes J, Tsimberidou A, Estey E, Kantarjian H, Giles FJ. Pilot study of gemtuzumab ozogamicin, liposomal daunorubicin, cytarabine and cyclosporine regimen in patients with refractory acute myelogenous leukemia. *Leuk Res* 2003; **27**: 887-891.
16. Tsimberidou AM, Estey E, Cortes JE, Garcia-Manero G, Faderl S, Verstovsek S, Thomas DA, Ferrajoli A, Keating MJ, O'Brien S, Kantarjian HM, Giles FJ. Mylotarg, fludarabine, cytarabine (ara-C), and cyclosporine (MFAC) regimen as post-remission therapy in acute myelogenous leukemia. *Cancer Chemother Pharmacol* 2003; **52**: 449-452.
17. Linenberger ML, Hong T, Flowers D, Sievers EL, Gooley TA, Bennett JM, Berger MS, Leopold LH, Appelbaum FR, Bernstein ID. Multidrug-resistance phenotype and clinical responses to gemtuzumab ozogamicin. *Blood* 2001; **98**: 988-994.
18. Walter RB, Raden BW, Hong TC, Flowers DA, Bernstein ID, Linenberger ML. Multidrug resistance protein attenuates gemtuzumab ozogamicin-induced cytotoxicity in acute myeloid leukemia cells. *Blood* 2003; **102**: 1466-1473.

PART 4

Concluding remarks and future perspectives

Detection of minimal residual disease (MRD) at different time-points during follow-up in myeloid leukemia patients provides information on the effectiveness of treatment and might enable the identification of risk groups. To evaluate therapy efficacy both flow cytometric and molecular PCR techniques can be used. In this thesis, we focussed on the further identification of potential PCR-based MRD targets, both in acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) patients. Additionally, we showed that quantitative MRD techniques are feasible and applicable in AML and CML patients for clinical decision-making during and after treatment.

What is the preferred method for evaluating therapy efficacy in myeloid leukemia?

Acute myeloid leukemia

In AML both immunological and molecular PCR techniques are used to evaluate therapy efficacy. These approaches provide quantitative MRD information during and after therapy, and allow the early identification of patients at high risk of relapse, thereby providing a window for therapeutic intervention. To minimize the chance of false-negative results (e.g. due to clonal evolution or phenotypic shifts), it would be attractive to monitor MRD by measuring two entirely independent features of the leukemic cells. However, currently not all AML patients can be evaluated with both techniques, due to the absence of leukemia-associated immunophenotypes (LAPs) or due to the lack of a specific or stable PCR-target. In addition, both approaches reach different sensitivities and have their specific limitations.

For real-time quantitative PCR (RQ-PCR) MRD analyses, standardized protocols for the most frequent fusion gene transcripts in AML are available, allowing to exchange and compare data between different centers.[1] Other advantages of this approach are the high sensitivity (10^{-4}/10^{-6}) and the stability of the targets between diagnosis and relapse. However, we showed that the stability over-time of fusion gene transcripts can vary substantially between patients and that the rate of degradation can differ between different types of transcripts and between peripheral blood (PB) and bone marrow (BM), if samples are not processed immediately after sampling.[2] Additionally, PCR-based MRD detection of chromosome aberrations with fusion genes is only applicable in a minority of AML patients (20-30%), which stresses the need for other PCR-targets. We showed that cross-lineage Ig/TCR gene rearrangements are generally complementary MRD-PCR targets to fusion genes in AML.[3] These Ig/TCR gene rearrangements might be useful as additional MRD-PCR targets, but they are only present in a small number of AML patients (<10%). In addition, it mainly concerns immature rearrangements, which can be subject to clonal evolution during therapy or at the time of relapse. We also showed that the *RAG1* and *RAG2* mRNA levels are not a straightforward tool for

the prediction of Ig/TCR gene rearrangements in AML. Therefore, the identification and characterization of Ig/TCR gene rearrangements, as well as the development of patient-specific primer/probe sets, makes it probably too time consuming for their use in a routine diagnostic laboratory. Finally, other molecular markers including *FLT3* mutations, *WT1* expression, and *MLL*-PTD may be targets for PCR-based MRD detection in a large number of AML patients.[4-7] However, contradictory data for their clinical use as follow-up markers are reported. These discrepancies may be due to different patients cohorts, the use of different primers covering different parts of the gene, or due the different PCR protocols. It illustrates the need for further standardization of these assays (e.g. within the European AML Network (LeukemiaNet)) in order to determine their technical feasibility and clinical applicability as MRD-markers in AML. In the meanwhile, the need for novel PCR-based MRD targets in AML are eagerly awaited; gene expression profiling using DNA micro-arrays may play a role in their identification.

Flow cytometric immunophenotyping in AML is based on the presence of LAPs and is applicable in the vast majority of AMLs (>80%).[8,9] The sensitivity of flow cytometry for MRD detection in clinical samples usually ranges from 10^{-3} to 10^{-4}. Although it is technically not a problem to evaluate 10^6 cells by flow cytometry, the difficulty is the recognition of small numbers of residual leukemic cells within the background of normal immature and maturating myelo-monocytic BM cells. This requires a detailed knowledge of the myelo-monocytic differentiation patterns, as well as insight in the specificity of LAPs, e.g. their occurrence in normal and regenerating BM samples. Standardization of flow cytometric MRD procedures including pre-analytical (e.g. selection of antibodies, sample preparation), analytical (e.g. number of cells analyzed) and post-analytical (e.g. identification of LAPs, expression of MRD data) steps are essential before MRD data obtained from different laboratories become comparable and interchangeable, and before MRD data can be implemented into treatment protocols with MRD-based therapeutic interventions. These harmonization processes are currently a subject of a Dutch-Belgian working group.

Overall, if a LAP can be identified in addition to a PCR target, it is advisable to use both flow cytometry and PCR analysis for monitoring MRD. This was illustrated in our patient with an inv(16)+ AML relapsing as an inv(16)+ ALL; the relapse was only predicted by PCR analysis, while flow cytometry gave false-negative results due to phenotypic changes.[10]

Chronic myeloid leukemia

MRD monitoring in CML patients is currently mainly done by RQ-PCR of *BCR-ABL* fusion gene transcripts. This approach is applicable in almost all CML patients and especially used for patients treated with imatinib or patients after transplantation.[11] A potential disadvantage of using mRNA targets for PCR analysis is the possibility of false-positive results due to contamination. However, the currently available closed

PCR systems have drastically reduced the possibility of contamination. While fusion transcripts are leukemia-specific targets for PCR-based MRD monitoring, genomic DNA breakpoint fusion sites differ in each CML thereby representing patient-specific targets. We identified the DNA sequences of *BCR-ABL* fusions, particularly for the $p230^{BCR\text{-}ABL}$ gene.[12] By using an allele-specific reverse primer positioned at the breakpoint area of the fusion genes, in combination with a μ-BCR-specific forward primer and probe, these patient-specific DNA breakpoint fusion sites may be attractive PCR targets for MRD by RQ-PCR. Although patient-specific DNA targets have several advantages over mRNA targets (e.g. low degradation rate, easy quantification, stability throughout the disease course), special methods are needed for their identification. For CML patients presenting in a blast crisis, flow cytometric immunophenotyping should be used in parallel to RQ-PCR.

What is the preferential sample type for clinical MRD monitoring in myeloid leukemia?

Both BM and PB samples can potentially be used for flow cytometric and molecular MRD monitoring in myeloid leukemia patients. Although the rather invasive and traumatic BM sampling procedure -especially for pediatric patients- might be an argument in favor of PB sampling, in clinical settings the required sensitivity for MRD detection will play an important role in the determination of the preferred sample type.

Chronic myeloid leukemia

Studies in CML patients showed that MRD levels of *BCR-ABL* transcripts analyzed by RQ-PCR seemed comparable in PB and BM samples. In the imatinib era, RQ-PCR data obtained from PB samples have shown to provide clinical prognostic MRD information; results from the IRIS study demonstrated that the reduction of *BCR-ABL* transcript levels in PB samples can predict the probability of remaining progression free.[13]

Acute myeloid leukemia

For RQ-PCR analysis of fusion gene transcripts in AML patients, it is still not clear whether PB might be a reliable alternative for BM in clinical MRD settings. Studies that demonstrated a prognostic significance of RQ-PCR analysis of fusion genes in AML mainly analyzed BM samples. Quantitative data on PB samples are scarce. For monitoring *PML-RARA* fusion gene transcripts, PB was shown to be only slightly less effective than BM.[14] Limited data comparing the transcript levels of *AML1-ETO* fusion genes during follow-up, showed that MRD levels in BM samples were generally higher than in the corresponding PB samples, with variable ranges between the two sample types.[15,16] We found comparable data for *CBFB-MYH11*

positive AML patients.[17] Nevertheless, results in AML with *AML1-ETO*, *CBFB-MYH11* and *PML-RARA* suggest that RQ-PCR analysis of PB samples might have clinical and prognostic value, especially if carried out at frequent time intervals. While an interval of less than 3 months has been suggested, the optimal frequency of MRD measurements as well as the clinical significance of detecting MRD in the PB and its relation to MRD detected in the BM still requires further investigation. Depending on the results of such studies, monitoring schedules for BM and/or PB samples after induction and consolidation might be adapted for each individual marker. If shown to be equally informative, PB could be preferred.

What can we expect from new techniques in the clinical management of patients care?

Gene expression profiling using DNA micro-array analysis

AML is an heterogeneous disease with distinct biological and prognostic groups; its accurate classification is critical for treatment and prognosis. In routine clinical practice, AML is currently analyzed and classified on the basis of its morphological and immunohistochemical characteristics, its cell surface marker expression, and molecular and cytogenetic abnormalities. So far, cytogenetics and molecular analyses provide the most important markers for determining prognosis and guiding therapy in AML. However, both techniques still provide far from complete insight into the diversity of AML, particularly for AML with a normal karyotype.

Results of gene expression profiling studies using DNA micro-arrays showed that this approach can successfully be applied to classify a variety of hematopoietic malignancies, including (subsets of) AML (Table 1).[18-21] The gene signatures have proven to be robust, showing diagnostic accuracies that in many cases exceed those achieved using routine diagnostic approaches. Gene expression profiling using DNA micro-arrays can capture much of the information assayed by the various 'classical' techniques, but importantly, gene profiling using DNA micro-arrays can broaden the scope of analysis, thereby filling important gaps in the diagnostic approach to AML. Gene expression profiling using DNA micro-arrays may:

1) identify clinically relevant outcome predictors (prognostic and disease progression makers), thereby supporting accurate risk-stratification of patients;[22]
2) provide information about drug sensitivity, thereby predicting response to therapy and allowing individualized selection of effective drugs;
3) provide insight into the critical molecular pathways that sustain the growth and survival of leukemic cell transformation, which might help in the development of targeted therapies.

A challenge for the near future will be, how to apply the micro-array technology and information from micro-array based experiments to clinical diagnostics. While

Table 1. AML-subtypes identified by gene expression profiling.

Classification into/identification of previously cytogenetically/molecular defined subgroups[18-21]
− t(15;17)
− t(8;21)
− inv(16)
− 11q23 abnormalities
− AML with normal karyotype
− *FLT3* mutations
− *CEBPA* mutations
− overexpression of *EVI1*
Identification of new subgroups/clusters
− new good and poor outcome subgroups within AML with normal karyotype, t(8;21) and inv(16)[19]
− cases classified as FAB AML-M7[20]
− novel clusters including FAB AML-M4/M5[18]

expression profiling continues to improve our understanding of hematological malignancies, some applications of micro-array based research might have the potential to enter clinical practice. Small arrays with strong predictive capabilities and arrays with the potential to recognize additional subgroups, might potentially be used in a clinical setting. It is also reasonable to expect that the discovery of novel diagnostic and prognostic markers by gene expression profiling will result in improved diagnosis and molecular tailored therapeutic plans for each individual patient. Future will show whether gene expression profiling will replace the 'classical' techniques or that all techniques evolve together as complementary approaches to improve diagnosis and therapy.

Proteomics

Another challenging technology with potential clinical applications in leukemia patients involves proteomics. Proteins are functionally the most relevant components of biological systems and form the basis of every conceivable cellular function. Changes observed in the proteome between leukemia patients and healthy individuals can be used as a biomarker to detect disease, and as a basis for the development of pharmacological drugs for therapeutic intervention.

Preliminary results from proteomic studies on leukemia show that distinct protein profiles of acute leukemia FAB subtypes can be identified.[23] It can be expected that proteomics might also identify additional subgroups of acute leukemia with clinical relevance.

Although the clinical applications of proteomics offers promises, further work is required to enhance the performance and reproducibility of established proteomic tools. Issues regarding the pre-analytical variables and analytical variability must be addressed to gain further progress.

What can we expect from new targeted drugs in myeloid leukemia?

Specific molecular targeted therapies including ATRA and imatinib mesylate have drastically improved the outcome of APL and CML patients, respectively.[13,24] We showed that even in patients with advanced CML persisting after allogeneic BM transplantation, imatinib mesylate can induce a durable complete remission.[25] Also new developments in monoclonal antibody therapy (e.g. Mylotarg®) have improved outcome for AML patients.[26] However, despite these (major) improvements, still many myeloid leukemia patients suffer from resistant disease or relapse, and especially the treatment of the older AML patients (age >60 years) is very disappointing with only modest, if any, improvement in overall survival over the past 3 to 4 decades.

Recent advances in the understanding of the genetic basis of myeloid leukemia and the nature of the mutations that contribute to the phenotype, have led to development of an expanding group of new targeted agents. Table 2 shows some of the potential new (molecular) targeted agents currently undergoing evaluation for the treatment of AML and CML. Phase I and/or II trials showed that these agents have clinical activity (e.g. reduction of blast cells in BM and PB and induction of partial or complete remission). However, the new drugs do not induce frequent and/or durable remission and long-term survival when administered as single agents. To obtain high(er) remission rates as well as durable remission, targeting the multiple pathophysiological pathways in myeloid leukemia is likely to be essential. Therefore, future clinical trials should use the new targeted drugs in combination with standard chemotherapy or should combine several new agents targeting more than one gene mutation, in order to prevent the development of resistance to any particular drug.

CONCLUSIONS

Our study has contributed to the further identification of PCR-based MRD targets in myeloid leukemia and has shown that quantitative approaches (flow cytometry and molecular techniques) can be applied in routine clinical diagnostics to evaluate therapy efficacy and to guide therapeutic interventions in myeloid leukemia.

Future AML protocols (e.g. HOVON protocols) aim to incorporate MRD data for MRD-based therapeutic interventions. It is to expect that this might improve patient outcome due to a more individualized risk-adapted strategy: treatment intensification for patients at high risk for relapse or treatment reduction for those with a low risk for relapse. A prior aim should be the further standardization of ‘classical’ MRD techniques such as flow cytometric approaches. In parallel, novel technologies such as gene expression profiling by DNA micro-array and proteomic analysis,

Table 2. Potential new (molecular) targeted agents for treatment of AML and CML.

	Targets	New drugs	Examples	Comments
AML	*RAS*	Farnesyltransferase inhibitors	R115777, tipifarnib, Zarnestra®	− overall response rate of 33% (complete and partial responses) when used as first-line in elderly poor-risk AML[27,28] − responses do not correlate with mutational status of *RAS* − role as adjunct to standard therapy under investigation
	FLT3-ITD	FLT3 tyrosine kinase inhibitors	CEP-701 PKC-412 SU11248 MLN518	− each of the inhibitors has activity in relapsed or refractory AML with *FLT3* activating mutations[29-31] − current clinical trials: *FLT3* inhibitors in combination with chemotherapy
	c-KIT	ATP competitive tyrosine kinase inhibitors	STI571, imatinib mesylate, Gleevec®	− overall hematologic response of 14% in refractory c-kit positive AML or AML not eligible for conventional chemotherapy[32] − D816V and D816Y mutations in the c-kit activation loop in AML are resistant to imatinib mesylate (but sensitive to PKC-412)[33]
	Histone deacetylase	Histone deacetylase (HDAC) inhibitors	suberoylanilide hydroxyamic acid (SAHA) (phenyl-)butyrate valproic acid (VPA) depsipetide	− induce differentiation of malignant cells[34,35] − show some activity in relapsed or refractory AML
	26S proteasome	Proteasome inhibitors	PS341, bortezomib, Velcade®	− approved drug for multiple myeloma − stabilizes proteins e.g. tumor-suppressor and pro-apoptotic proteins[36] − demonstrated preclinical synergistic activity with HDAC inhibitors and potential single-agent activity in leukemia[37] − currently evaluated in combination with chemotherapy
	VEGF	Antiangiogenesis agents	bevacizumab	− potential role because of increased bone marrow vascularization in AML at diagnosis[38] − currently evaluated in combination with chemotherapy in newly AML
	BCL2-protein	BCL-2 antisense oligonucleotide	G3139	− down-regulation of bcl-2 may restore chemosensitivity to leukemia cells[39] − in combination with chemotherapy: complete remission rate of 35% in refractory or relapsed AML[40]
CML	*BCR-ABL*	ATP competitive tyrosine kinase inhibitors	BMS-354825, dasatinib AMN107	− clinical activity in CML resistant to imatinib mesylate[41,42] − sensitivities against all *BCR-ABL* point mutations except for T315I mutation[42]
		non-ATP competitive inhibitors		− *in vitro* activity against all imatinib mesylate resistant mutants, including T315I mutations[43,44]

VEGF: vascular endothelial growth factor

should focus on 1) the further identification of new MRD targets, 2) the identification of clinically useful risk gene expression signatures, and 3) the development of new targeted therapies.

REFERENCES

1. Gabert J, Beillard E, Van der Velden VHJ, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cave H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, Gonzalez M, Viehmann S, Malec M, Saglio G, Van Dongen JJM. Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) of fusion gene transcripts for minimal residual disease detection in leukemia - A Europe Against Cancer Program. *Leukemia* 2003; **12**: 2318-2357.
2. Van der Velden VHJ, Boeckx N, Gonzales M, Malec M, Barbany G, Lion T, Gottardi E, Pallisgaard N, Beillard E, Hop WCJ, Hoogeveen PG, Gabert J, Van Dongen JJM on behalf of the Europe Against Cancer Program. Differential stability of control gene and fusion gene transcripts over time may hamper accurate quantification of minimal residual disease – a study within the Europe Against Cancer Program. *Leukemia* 2004; **18**: 983-988.
3. Boeckx N, Willemse MJ, Szczepanski T, Van der Velden VHJ, Langerak AW, Vandekerckhove P, Van Dongen JJM. Fusion gene transcripts and Ig/TCR gene rearrangements are complementary but infrequent targets for PCR-based detection of minimal residual disease in acute myeloid leukemia. *Leukemia* 2002; **16**: 368-375.
4. Weisser M, Kern W, Schoch C, Hiddemann W, Haferlach T, Schnittger S. Risk assessment by monitoring expression levels of partial tandem duplications in the *MLL* gene in acute myeloid leukemia during therapy. *Haematologica* 2005; **90**: 881-889.
5. Schnittger S, Schoch C, Kern W, Hiddemann W, Haferlach T. *FLT3* length mutations as marker for follow-up studies in acute myeloid leukaemia. *Acta Haematol* 2004; **112**: 68-78.
6. Scholl S, Loncarevic IF, Krause C, Kunert C, Clement JH, Hoffken K. Minimal residual disease based on patient specific *FLT3*-ITD and -ITT mutations in acute myeloid leukemia. *Leuk Res* 2005; **29**: 849-853.
7. Ostergaard M, Olesen LH, Hasle H, Kjeldsen E, Hokland P. *WT1* gene expression: an excellent tool for monitoring minimal residual disease in 70% of acute myeloid leukaemia patients - results from a single-centre study. *Br J Haematol* 2004; **125**: 590-600.
8. Kern W, Danhauser-Riedl S, Ratei R, Schnittger S, Schoch C, Kolb HJ, Ludwig WD, Hiddemann W, Haferlach T. Detection of minimal residual disease in unselected patients with acute myeloid leukemia using multiparameter flow cytometry for definition of leukemia-associated immunophenotypes and determination of their frequencies in normal bone marrow. *Haematologica* 2003; **88**: 646-653.
9. Voskova D, Valekova L, Fedorova J, Hudecek J, Kubisz P. Leukemic cells and aberrant phenotypes in acute leukemia patients: a flow cytometry analysis. *Neoplasma* 2003; **50**: 422-427.
10. Boeckx N, Van der Velden VHJ, Boogaerts M, Hagemeijer A, Vandenberghe P, Van Dongen JJM. An inv(16)(p13q22) positive acute myeloid leukaemia relapsing as acute precursor B-cell lymphoblastic leukaemia. *Haematologica* 2004; **89**: ECR28.
11. Oehler VG, Radich JP. Monitoring *BCR-ABL* by polymerase chain reaction in the treatment of chronic myeloid leukemia. *Curr Oncol Rep* 2003; **5**: 426-435.
12. Boeckx N, Jansen MWJC, Haskovec C, Vandenberghe P, Van der Velden VHJ, Van Dongen JJM. Identification of e19a2 *BCR-ABL* fusions (µ-BCR breakpoints) at the DNA level by ligation-mediated PCR. *Leukemia* 2005; **19**: 1292-1295.

13. Guilhot F. Sustained Durability of Responses Plus High Rates of Cytogenetic Responses Result in Long-Term Benefit for Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia (CML-CP) Treated with Imatinib (IM) Therapy: Update from the IRIS Study. *Blood* 2004; **104**: 21a (abstract).
14. Gallagher RE, Yeap BY, Bi W, Livak KJ, Beaubier N, Rao S, Bloomfield CD, Appelbaum FR, Tallman MS, Slack JL, Willman CL. Quantitative real-time RT-PCR analysis of *PML-RAR*alpha mRNA in acute promyelocytic leukemia: assessment of prognostic significance in adult patients from intergroup protocol 0129. *Blood* 2003; **101**: 2521-2528.
15. Leroy H, Botton SD, Grardel-Duflos N, Darre S, Lelei X, Roumier C, Morschhauser F, Lai JL, Bauters F, Fenaux P, Preudhomme C. Prognostic value of real-time quantitative PCR (RQ-PCR) in AML with t(8;21). *Leukemia* 2005; **19**: 367-372.
16. Tobal K, Newton J, Macheta M, Chang J, Morgenstern G, Evans PA, Morgan G, Lucas GS, Liu Yin JA. Molecular quantitation of minimal residual disease in acute myeloid leukemia with t(8;21) can identify patients in durable remission and predict clinical relapse. *Blood* 2000; **95**: 815-819.
17. Boeckx N, De Roover J, Van der Velden VHJ, Maertens J, Uyttebroeck A, Vandenberghe P, Van Dongen JJM. Quantification of *CBFB-MYH11* fusion gene levels in paired peripheral blood and bone marrow samples by real-time PCR. *Leukemia* 2005; **19**: 1988-1990.
18. Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh Van Waalwijk Van Doorn-Khosrovani S, Boer JM, Beverloo HB, Moorhouse MJ, Van der Spek PJ, Löwenberg B, Delwel R. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* 2004; **350**: 1617-1628.
19. Bullinger L, Dohner K, Bair E, Frohling S, Schlenk RF, Tibshirani R, Dohner H, Pollack JR. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med* 2004; **350**: 1605-1616.
20. Ross ME, Mahfouz R, Onciu M, Liu HC, Zhou X, Song G, Shurtleff SA, Pounds S, Cheng C, Ma J, Ribeiro RC, Rubnitz JE, Girtman K, Williams WK, Raimondi SC, Liang DC, Shih LY, Pui CH, Downing JR. Gene expression profiling of pediatric acute myelogenous leukemia. *Blood* 2004; **104**: 3679-3687.
21. Haferlach T, Kohlmann A, Schnittger S, Dugas M, Hiddemann W, Kern W, Schoch C. Global approach to the diagnosis of leukemia using gene expression profiling. *Blood* 2005; **106**: 1189-1198.
22. Yagi T, Morimoto A, Eguchi M, Hibi S, Sako M, Ishii E, Mizutani S, Imashuku S, Ohki M, Ichikawa H. Identification of a gene expression signature associated with pediatric AML prognosis. *Blood* 2003; **102**: 1849-1856.
23. Cui JW, Wang J, He K, Jin BF, Wang HX, Li W, Kang LH, Hu MR, Li HY, Yu M, Shen BF, Wang GJ, Zhang XM. Proteomic analysis of human acute leukemia cells: insight into their classification. *Clin Cancer Res* 2004; **10**: 6887-6896.
24. Tallman MS, Andersen JW, Schiffer CA, Appelbaum FR, Feusner JH, Woods WG, Ogden A, Weinstein H, Shepherd L, Willman C, Bloomfield CD, Rowe JM, Wiernik PH. All-*trans* retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup protocol. *Blood* 2002; **100**: 4298-4302.
25. Vandenberghe P, Boeckx N, Ronsyn E, Decorte R, Verhoef G, Hagemeijer A. Imatinib mesylate induces durable complete remission of advanced CML persisting after allogeneic bone marrow transplantation. *Leukemia* 2003; **17**: 458-460.
26. Larson RA, Boogaerts M, Estey E, Karanes C, Stadtmauer EA, Sievers EL, Mineur P, Bennett JM, Berger MS, Eten CB, Munteanu M, Loken MR, Van Dongen JJM, Bernstein ID, Appelbaum FR. Mylotarg Study Group. Antibody-targeted chemotherapy of older patients with acute myeloid leukemia in first relapse using Mylotarg (gemtuzumab ozogamicin). *Leukemia* 2002; **16**: 1627-1636.
27. Harrousseau JL, Stone R, Thomas X, Lancet J, Arani R, Thibault A, Rackoff W. Interim results from a phase II study of R115777 (Zarnestra) in patients with relapsed and refractory acute myelogenous leukaemia. *Proc Am Soc Clin Oncol* 2002; **21**: 265a (abstract).

28. Lancet JE, Gotlib J, Gojo I, Feldman EJ, Morris L, Thibault A, Liesveld JL, Greer J, Dugan K, Raponi M, Wright JJ, Greenberg PL, Karp JE. Tipifarnib (ZARNESTRA™) in previously untreated poor-risk AML of the elderly: updated results of a multicenter phase 2 trial. *Blood* 2004; **104**: 874a (abstract).
29. Smith BD, Levis M, Beran M, Giles F, Kantarjian H, Berg K, Murphy KM, Dauses T, Allebach J, Small D. Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. *Blood* 2004; **103**: 3669-3676.
30. Stone RM, DeAngelo DJ, Klimek V, Galinsky I, Estey E, Nimer SD, Grandin W, Lebwohl D, Wang Y, Cohen P, Fox EA, Neuberg D, Clark J, Gilliland DG, Griffin JD. Patients with acute myeloid leukemia and an activating mutation in *FLT3* respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood* 2005; **105**: 54-60.
31. Fiedler W, Serve H, Dohner H, Schwittay M, Ottmann OG, O'Farrell AM, Bello CL, Allred R, Manning WC, Cherrington JM, Louie SG, Hong W, Brega NM, Massimini G, Scigalla P, Berdel WE, Hossfeld DK. A phase 1 study of SU11248 in the treatment of patients with refractory or resistant acute myeloid leukemia (AML) or not amenable to conventional therapy for the disease. *Blood* 2005; **105**: 986-993.
32. Kindler T, Breitenbuecher F, Marx A, Beck J, Hess G, Weinkauf B, Duyster J, Peschel C, Kirkpatrick CJ, Theobald M, Gschaidmeier H, Huber C, Fischer T. Efficacy and safety of imatinib in adult patients with c-kit-positive acute myeloid leukemia. *Blood* 2004; **103**: 3644-3654.
33. Growney JD, Clark JJ, Adelsperger J, Stone R, Fabbro D, Griffin JD, Gilliland DG. Activation mutations of human c-KIT resistant to imatinib mesylate are sensitive to the tyrosine kinase inhibitor PKC412. *Blood* 2005; **106**: 721-724.
34. Kosugi H, Towatari M, Hatano S, Kitamura K, Kiyoi H, Kinoshita T, Tanimoto M, Murate T, Kawashima K, Saito H Naoe T. Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. *Leukemia* 1999; **13**: 1316-1324.
35. Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, Heinzel T. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* 2001; **20**: 6969-6978.
36. Boccadoro M, Morgan G, Cavenagh J. Preclinical evaluation of the proteasome inhibitor bortezomib in cancer therapy. *Cancer Cell Int* 2005; **5**: 18-26.
37. Yu C, Rahmani M, Conrad D, Subler M, Dent P, Grant S. The proteasome inhibitor bortezomib interacts synergistically with histone deacetylase inhibitors to induce apoptosis in *BCR/ABL*+ cells sensitive and resistant to STI571. *Blood* 2003; **102**: 3765-3774.
38. De Bont ES, Rosati S, Jacobs S, Kamps WA, Vellenga E. Increased bone marrow vascularization in patients with acute myeloid leukaemia: a possible role for vascular endothelial growth factor. *Br J Haematol* 2001; **113**: 296-304.
39. Konopleva M, Tari AM, Estrov Z, Harris D, Xie Z, Zhao S, Lopez-Berestein G, Andreeff M. Liposomal Bcl-2 antisense oligonucleotides enhance proliferation, sensitize acute myeloid leukemia to cytosine-arabinoside, and induce apoptosis independent of other antiapoptotic proteins. *Blood* 2000; **95**: 3929-3938.
40. Marcucci G, Byrd JC, Dai g, Klisovic MI, Kourlas PJ, Young DC, Cataland SR, Fisher DB, Lucas D, Chan KK, Porcu P, Lin Z, Farag SF, Frankel SR, Zwiebel JA, Kraut EH, Balcerzak SP, Bloomfield CD, Grever MR, Caligiuri MA. Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukaemia. *Blood* 2003; **101**: 425-432.
41. Talpaz M, Kantarjian H, Shah NP, Donato N, Nicoll J, Bai SA, Huang F, Clark E, DeCillis AP, Sawyers C. Hematologic and Cytogenetic Responses in Imatinib-Resistant Accelerated and Blast Phase Chronic Myeloid Leukemia (CML) Patients Treated with the Dual SRC/ABL Kinase Inhibitor BMS-354825: Results from a Phase I Dose Escalation Study. *Blood* 2004; **104**: 20a (abstract).

42. Mestan J, Weisberg E, Cowan-Jacob S, Fabbro D, Furet P, Fendrich G, Goutte Ge, Kempf D, Gaugler M, Griffin DJ, Manley WP. AMN107: *In vitro* profile of a new inhibitor of the tyrosine kinase activity of BCR-ABL. *Blood* 2004; **104**: 1978a (abstract).
43. Ahmad K. New agent overcomes resistance to imatinib. *Lancet Oncol* 2005; **6**: 137.
44. Gumireddy K, Baker SJ, Cosenza SC, John P, Kang AD, Robell KA, Reddy MV, Reddy EP. A non-ATP-competitive inhibitor of BCR-ABL overrides imatinib resistance. *Proc Natl Acad Sci USA* 2005; **102**: 1992-1997.

ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AP	acceleration phase
APL	acute promyelocytic leukemia
ATO	arsenic trioxide
ATP	adenosine triphosphate
ATRA	all-*trans*-retinoic acid
AUL	acute undifferentiated leukemia
BC	blast crisis
BCR	breakpoint cluster region
BM	bone marrow
C gene segment	constant gene segment
CCyR	complete cytogenetic response
CD	cluster of differentiation
CML	chronic myeloid leukemia
CP	chronic phase
CR	complete remission
CR1	first complete remission
C_T	threshold cycle
D gene segment	diversity gene segment
DFS	disease free survival
DNA	deoxyribonucleic acid
EAC	Europe Against Cancer
FAB	French-American-British
FDA	Food and Drug Administration
FICTION	fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms
FISH	fluorescent *in situ* hybridization
GO	gemtuzumab ozogamicin
HOVON	stichting hemato oncologie voor volwassenen Nederland
HSC	hematopoietic stem cell
HU	hydroxyurea
IFN	interferon
Ig	immunoglobin
IGH	immunoglobin heavy chain gene
IGK	immunoglobin kappa light chain gene
IGL	immunoglobin lambda light chain gene
IM	imatinib mesylate
IRIS	International Randomized Study of Interferon and STI571
ITD	internal tandem duplications

J gene segment	joining gene segment
Kde	immunoglobin kappa deleting element
LAIP	leukemia associated immunophenotype
LM-PCR	ligation mediated polymerase chain reaction
MNC	mononuclear cells
MPO	myeloperoxidase
MRD	minimal residual disease
PB	peripheral blood
PCR	polymerase chain reaction
Ph chromosome	Philadelphia chromosome
precursor B-ALL	precursor-B-cell acute lymphoblastic leukemia
RAG	recombination-activating gene
RNA	ribonucleic acid
RSS	recombination signal sequence
RT-PCR	reverse transcriptase polymerase chain reaction
RQ-PCR	real-time quantitative polymerase chain reaction
SB	Southern blotting
SCT	stem cell transplantation
T-ALL	T-cell acute lymphoblastic leukemia
TCR	T-cell receptor
TCRA	T-cell receptor alpha gene
TCRB	T-cell receptor beta gene
TCRD	T-cell receptor delta gene
TCRG	T-cell receptor gamma gene
TdT	terminal deoxynucleotidyl transferase
V gene segment	variable gene segment
WHO	World Health Organisation

SUMMARY

Acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) are malignancies characterized by an uncontrolled proliferation of (immature) myeloid cells in the bone marrow (BM). During the past decades, a lot of progress has been made in the treatment and the understanding of the biology of AML and CML. Patients with myeloid leukemia are usually treated with chemotherapy, possible followed by stem cell transplantation. New therapeutic agents are recently available including monoclonal antibodies and agents targeting the molecular basis of the disease (see Chapter 1). Despite therapy, many patients experience relapse and most of them will ultimately die of disease progression.

The response to therapy can be evaluated by a cytomorphologic examination of the BM. However, microscopic techniques can only detect 1 to 5% of malignant cells, which is not sufficiently sensitive for the identification of patients who are prone to relapse. Indeed, patients who reach a complete remission, cytomorphologically defined as the presence of less than 5% of blast cells in the BM, may ultimately relapse. To trace low frequencies of remaining leukemic cells, i.e. minimal residual disease (MRD), more sensitive methods are required. MRD techniques should allow the detection of one leukemic cell within the background of at least 10^4 normal cells (sensitivity of $\leq 10^{-4}$).

The first part of our study focussed on polymerase chain reaction (PCR) detectable MRD-targets. These MRD targets represent DNA and/or RNA sequences of the leukemic cells that are assumed to be unique for each patient or leukemia, as they result from 1) chromosome aberrations with the formation of fusion genes, or 2) immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements. The unique "*fingerprint-like*" sequences can easily be identified and characterized by PCR-analysis and sequencing. Moreover, "real-time quantitative PCR" (RQ-PCR) allows not only the detection of residual leukemic cells, but also the level of MRD.

In Chapter 2.2, we investigated the occurrence of "cross-lineage" Ig/TCR gene rearrangements in a large group of AML patients. Although the frequency of PCR-detectable Ig/TCR gene rearrangements appeared to be low (<10%), Ig/TCR gene rearrangements were complementary to the presence of fusion gene transcripts that were found in 16% of AML patients. This implies that "cross-lineage" Ig/TCR gene rearrangements are complementary MRD-PCR targets in AML patients and that PCR allows sensitive MRD detection in 20-25% of AML patients. Because Ig/TCR gene rearrangements were rather infrequent, we analyzed whether high expression levels of *RAG* transcripts (enzymes involved in the process of Ig/TCR gene rearrangements) correlated with the presence of the "cross-lineage" Ig/TCR gene rearrangements in AML. Since no correlation was found, analysis of *RAG* expression levels can not be used as a screening tool for predicting the presence of Ig/TCR gene rearrangements in AML.

In Chapter 2.3, we focused on the over-time stability of fusion gene transcripts as MRD-PCR targets. Shipment of freshly collected samples to the PCR laboratory may take considerable time. During this period, the quality of the RNA present in the samples may deteriorate. We showed that fusion gene transcripts as well as control gene transcripts rapidly degrade *ex vivo* and that the rate of degradation can differ between different types of transcripts, between peripheral blood (PB) and BM, and between patients. As such differential degradation may result in an over- or underestimation of MRD levels, samples should preferably be processed on the day of sampling.

In Chapter 2.4, we characterized the rare *µBCR-ABL* fusion gene transcript at the DNA level. Fusion gene transcripts are most frequently analysed at the RNA level, but such PCR products are *leukemia-specific*, not *patient-specific*. Consequently, a potential disadvantage of monitoring fusion gene transcripts at the RNA level, is the possibility of cross-contamination. Using a ligation-mediated PCR, we identified the *µBCR-ABL* fusion sites in three samples. The identified DNA sequences represent patient-specific "*fingerprints*" and can potentially be used as PCR targets for MRD monitoring. We also showed that the breakpoints were rather clustered within the genome.

In the second part of our study we focused on the clinical applicability of MRD analyses for the evaluation of therapy-efficacy in myeloid leukemias. Both flow cytometric and molecular techniques are used for the detection and quantification of MRD. Flow cytometric analysis is based on the detection of so-called "leukemia-associated phenotypes" (LAPs) that are absent or extremely uncommon within normal BM. These LAPs are found in about 80% of AML patients and can be used during follow-up to discriminate between residual leukemic and normal cells.

In routine clinical practice, both flow cytometric and PCR analyses are performed on diagnostic AML samples. If at diagnosis a LAP is identified in addition to a PCR-target, it is recommended to monitor AML patients during and after therapy with both MRD-techniques. This is illustrated in Chapter 3.2. Herein we report on a patient with a immunophenotypic change of the leukemic cells between diagnosis (AML) and relapse (acute lymphoblastic leukemia), while the *CBFB-MYH11* fusion gene transcript remained stable. If only flow cytometric immunophenotyping was used to monitor this patient, the relapse would only have been detected at the moment of clinical relapse. For certain AML subtypes (e.g. AML-M3), it has been proven that treatment intervention at the time of molecular relapse may result in better clinical outcome than treatment intervention at the time of overt clinical relapse. Further studies need to investigate whether treatment intervention at the time of molecular relapse can also improve outcome in *CBFB-MYH11* positive AML patients.

In Chapter 3.3 we evaluated the possibility to safely replace BM sampling by PB sampling for MRD monitoring in *CBFB-MYH11* positive AML. RQ-PCR results showed that MRD levels in BM samples are generally higher than in PB samples (ranges: 0,8 to 100 fold). Nevertheless, monitoring of *CBFB-MYH11* transcript levels

in PB did have predictive value: MRD levels in PB predicted disease reoccurrence 2,5 to 4 months prior to the cytomorphological BM relapse. A time frame of at least 2,5 months should be enough to adjust therapy, thereby increasing the chance to reach a molecular remission.

The hallmark of CML is the presence of the Philadelphia chromosome resulting from a translocation between chromosome 9 and 22, with the formation of a *BCR-ABL* fusion gene and hybrid protein. This latter has a constitutively active tyrosine kinase activity, which induces aberrant oncogenic signalling. RQ-PCR allows the quantification of *BCR-ABL* fusion gene transcripts during therapy. To date allogeneic stem cell transplantation is the only treatment modality with an established curative treatment potential for CML. However, recently Gleevec® (imatinib mesylate), a selective inhibitor of the BCR-ABL tyrosine kinase, is routinely used for treatment of CML. Imatinib mesylate shows superior outcomes for newly diagnosed CML patients in chronic phase (i.e. the first stage of the disease), frequently with major cytogenetic and molecular responses. We showed that Gleevec® can also induce durable complete remission in a patient with advanced CML persisting after allogeneic BM transplantation (see Chapter 3.4).

During the last decades, the prognosis of AML in young patients (≤60 years of age) has been significantly improved: current treatment protocols induce a complete remission in 75-80% of patients with a 5-years survival of 30-40%. This is in contrast to the limited progress in outcome results in elderly AML patients (>60 years of age): complete remissions in 50-55% of patients with a 5-years overall survival of ~10%. This is partially explained by the limited ability of the older patients to tolerate intensive chemotherapy. Recently, new treatment modalities have become available including the use of monoclonal antibodies selectively targeting the leukemic cells, e.g. Mylotarg® (gemtuzumab ozogamicin). Mylotarg® is a humanized CD33 monoclonal antibody chemically linked to calicheamicin, a highly potent antitumor antibiotic. Mylotarg® targets the surface CD33 antigen that is found on >85% of AML cells. In elderly patients in first relapse, Mylotarg® induces a remission in 30% when administrated as two infusions at a dose of 9 mg/m^2. In Chapter 3.5 we show that after administration of these doses, the CD33 saturation in BM is significantly reduced (40-90%) as compared with the CD33 saturation in corresponding PB samples (>90%) and that these reduced CD33 saturation levels *in vitro* result in less efficient cell-kill. Our results suggested that the final CD33 saturation level in BM is not only affected by the peripheral CD33-antigen load, but may also be dependant on the total CD33-antigen load present in the BM. This implies that for efficient killing of AML blasts in BM, the effective gemtuzumab dose should be increased or alternatively, that the CD33 antigen load should be reduced prior to Mylotarg® administration, preferably by the design of treatment protocols that combine chemotherapy and Mylotarg®.

In conclusion, the results of our study have, 1) contributed to the further identification of PCR-detectable MRD targets in AML and CML, 2) showed that BM

sampling can be replaced by PB sampling for MRD monitoring in *CBFB-MYH11* positive AML, 3) illustrated that MRD-monitoring with PCR and flow cytometric techniques has clinical relevance, and 4) stressed the need for adequate evaluation of new (antibody)therapies for the design of future treatment protocols.

MRD-based treatment interventions are routinely used in the AML-M3 subtype and transplanted CML patients. Further studies are needed to evaluate whether such MRD-based treatment interventions (treatment intensification or reduction) can also improve overall outcome in other AML subtypes. In addition, further research for reliable MRD targets, but especially studies focussing on new therapeutics might significantly improve the overall outcome in myeloid leukemias.

SAMENVATTING

Acute myeloïde leukemie (AML) en chronische myeloïde leukemie (CML) zijn vormen van bloedkanker gekenmerkt door een ongecontroleerde proliferatie van (onrijpe) myeloïde cellen in het beenmerg. De afgelopen jaren is veel vooruitgang geboekt in de behandeling van myeloïde leukemie, alsook in de inzichten van de ontstaansmechanismen ervan. Patiënten met een myeloïde leukemie worden in het algemeen behandeld met chemotherapie, en eventueel een beenmergtransplantatie. Sinds kort bestaan er ook nieuwe therapeutische mogelijkheden zoals het gebruik van antistoffen en producten die selectief inwerken op het onderliggende moleculaire defect van de leukemie (zie Hoofdstuk 1). Ondanks behandeling komt de ziekte bij een deel van de patiënten terug (recidief), van wie uiteindelijk een groot deel zal overlijden aan de ziekte.

Door een cytomorfologisch (microscopisch) onderzoek van het beenmerg kan bepaald worden hoe een patiënt reageert op de therapie. Met microscopisch onderzoek kunnen echter slechts 1 tot 5% leukemiecellen worden gedetecteerd, hetgeen onvoldoende is voor het herkennen van patiënten die een grote kans hebben op een recidief. Immers bij een relatief groot deel van de patiënten bij wie na behandeling microscopisch geen leukemiecellen meer aantoonbaar zijn in het beenmerg (complete remissie), komt de ziekte toch terug. De kleine aantallen leukemiecellen, die ondanks therapie achterblijven, vormen de zogenaamde "minimale restziekte" ofwel "*minimal-residual disease*" (MRD). Het opsporen van MRD vereist zeer gevoelige technieken die bij voorkeur minimaal één leukemiecel tussen $1x10^4$ normale cellen (sensitiviteit $\leq 1x10^{-4}$) kunnen detecteren.

Het eerste deel van het onderzoek in dit proefschrift was gericht op MRD-targets die met behulp van de polymerase ketting reactie (PCR) kunnen worden opgespoord. Deze MRD-PCR targets zijn basepaarvolgordes (sequenties) van genetisch materiaal (DNA en/of RNA) die uniek zijn voor de leukemiecellen, omdat zij afkomstig zijn van (1) chromosomale afwijkingen met de vorming van fusiegentranscripten, of van (2) immunoglobuline (Ig) en T-celreceptor (TCR) genherschikkingen. Ze vormen dus een soort unieke "vingerafdruk" van de leukemiecellen. Met PCR kunnen ze op een relatief eenvoudige wijze worden geïdentificeerd en gekarakteriseerd. Door gebruik te maken van een kwantitatieve PCR methode, de zogenoemde "*real-time quantitative PCR*" (RQ-PCR) kan niet alleen MRD op een gevoelige manier worden gedetecteerd, maar bovendien kan ook het MRD niveau, dus het aantal leukemiecellen, worden gekwantificeerd.

In Hoofdstuk 2.2 onderzochten we het voorkomen van "cross-lineage" Ig/TCR genherschikkingen in een grote groep AML patiënten. Ig/TCR genherschikkingen die we tot op sequentieniveau konden karakteriseren, werden slechts in een klein deel (<10%) van de AML patiënten aangetroffen. Een belangrijke bevinding was echter dat deze Ig/TCR genherschikkingen veelal complementair zijn aan de aanwezigheid van fusiegentranscripten, die we bij 16% van de AML patiënten aantroffen.

Dit betekent dat “cross-lineage” Ig/TCR genherschikkingen een complementair MRD-PCR target vormen bij AML patiënten en dat met de PCR techniek ongeveer 20% tot 25% van de AML patiënten kunnen worden vervolgd voor de aanwezigheid van MRD. Omdat Ig/TCR genherschikkingen niet frequent worden gevonden bij AML patiënten, onderzochten we of hoge expressies van RAG transcripten (dit zijn enzymen die betrokken zijn in het proces van Ig/TCR genherschikkingen) de aanwezigheid van Ig/TCR genherschikkingen bij AML kunnen voorspellen. We konden echter geen correlatie tussen het expressieniveau van *RAG* en het voorkomen van “cross-lineage” Ig/TCR herschikkingen bij AML aantonen, zodat het *RAG* expressieniveau niet als screeningstest kan worden gebruikt voor het voorspellen van de aanwezigheid van Ig/TCR genherschikkingen in AML.

In Hoofdstuk 2.3 richtten we ons op fusiegentranscipten als MRD-PCR targets waarbij we vooral geïnteresseerd waren in de stabiliteit van deze PCR targets tussen staalafname en laboratoriumanalyse. Immers, de tijdspanne tussen het moment van staalafname voor PCR-analyses en het verwerken van het staal in gespecialiseerde laboratoria kan soms enkele dagen duren; tijdens deze transportperiode kan de kwaliteit van het RNA achteruitgaan. We toonden aan dat fusiegentranscripten snel afbreken *ex vivo*, maar belangrijker dat de mate van afbraak varieert tussen de verschillende soorten van fusiegentranscripten, tussen verschillende staalsoorten (beenmerg of perifeer bloed) en tussen patiënten. Dit impliceert dat het niveau van MRD mogelijk foutief wordt ingeschat en dat daarom stalen bijvoorkeur dezelfde dag moeten worden verwerkt.

In Hoofdstuk 2.4 hebben wij een zeldzame vorm van het *BCR-ABL* fusiegen, namelijk het *µBCR-ABL* fusiegen, op DNA niveau gekarakteriseerd. Meestal worden MRD PCR-analyses van fusiegenen op RNA niveau uitgevoerd (zoals wij ook deden in de Hoofdstukken 2.2 en 2.3). Fusiegentranscripten zijn weliswaar wel *leukemie-specifiek*, maar niet *patiënt-specifiek*, waardoor een contaminatie (besmetting van een negatief staal door een positief staal) nooit helemaal is uit te sluiten. Met een speciale PCR-techniek (ligatie-gemedieerde PCR) identificeerden we de exacte DNA breukpunten van de *µBCR-ABL* fusiegenen van drie verschillende leukemieën. Deze geïdentificeerde DNA codes vormen patiënt-specifieke “vingerafdrukken” die uitstekend bruikbaar zijn als MRD-PCR targets. We toonden bovendien aan dat de breukpunten relatief sterk geclusterd liggen in het genoom.

Het tweede deel van het onderzoek in dit proefschrift richtte zich op de klinische toepassingen van MRD analyses voor de evaluatie van de doeltreffendheid van de therapie. Naast PCR is ook flowcytometrische immunofenotypering geschikt om op een gevoelige manier MRD te detecteren en te kwantificeren. Flowcytometrische MRD analyse is gebaseerd op het opsporen van leukemie-geassocieerde fenotypes (“leukemia-associated phenotypes”: LAP’s) die niet of nauwelijks voorkomen in een normaal beenmerg. Deze LAP’s die in ~80% van de AML patiënten worden geïdentificeerd, kunnen worden gebruikt om gedurende en na de therapie de eventueel resterende leukemiecellen te onderscheiden van de normale cellen.

Bij de diagnose van een nieuwe AML worden steeds flowcytometrische en PCR analyses uitgevoerd. Indien hierbij zowel LAP's als MRD-PCR-targets worden gedetecteerd, is het aanbevolen om de AML tijdens en na therapie met de twee MRD technieken te vervolgen. De complementariteit van deze twee MRD technieken wordt geïllustreerd in Hoofdstuk 3.2, waarin we een patiënt rapporteren waarbij de fenotypische kenmerken (LAP's) van de leukemiecellen veranderden tussen de oorspronkelijke diagnose (AML) en zijn recidief (acute lymfoblastaire leukemie), terwijl de moleculaire afwijking (een *CBFB-MYH11* fusiegentranscript) stabiel bleef tussen diagnose en recidief. Indien men deze patiënt enkel zou hebben vervolgd met flowcytometrische technieken, zou het recidief pas laattijdig zijn gedetecteerd, namelijk op het moment van het klinische recidief. Voor bepaalde types van AML (bv. AML-M3) is reeds duidelijk aangetoond dat de prognose van de patiënt verbetert indien therapeutisch wordt ingegrepen op het moment dat een recidief met PCR of flowcytometrische technieken wordt gedetecteerd. Verdere prognostische studies moeten uitwijzen of dit ook geldt voor patiënten met een *CBFB-MYH11* positieve AML.

Hoofdstuk 3.3 vat de resultaten samen van een studie waarin we evalueerden of een perifeer bloedstaal een waardig alternatief kan vormen voor een beenmerg voor het vervolgen van MRD tijdens en na therapie. We focusten hierbij op AML patiënten met een *CBFB-MYH11* fusiegentranscript. RQ-PCR resultaten toonden enerzijds dat het niveau van MRD in beenmerg gewoonlijk hoger ligt dan in perifeer bloed (factor 0,8 tot 100), maar anderzijds ook dat MRD-analyses van perifeer bloed toch belangrijke prognostische informatie bevatten. Immers, RQ-PCR op perifeer bloed stelde ons in staat om recidieven vroegtijdig op te sporen, namelijk 2,5 tot 4 maanden vóór de patiënt een klinisch recidief vertoonde. Deze periode is vermoedelijk voldoende om de behandelend arts de tijd te geven om vroegtijdig de behandeling terug op te starten of om de therapie te intensiveren, waardoor de kans om opnieuw een moleculaire remissie te bereiken hopelijk wordt vergroot.

Hét kenmerk van een CML is de aanwezigheid van het Philadelphia chromosoom. Dit abnormale chromosoom is het resultaat van een translocatie tussen chromosomen 9 en 22, waarbij het *BCR-ABL* fusiegen ontstaat. Het *BCR-ABL* fusiegen leidt tot een continue doorgaande tyrosine kinase activiteit, die een belangrijke rol speelt in het ontstaan van CML. Met behulp van RQ-PCR technieken wordt het *BCR-ABL* fusiegentranscript tijdens de behandeling vervolgd om daarmee de effectiviteit van de behandeling te evalueren. Tot voor enkele jaren vormde een beenmergtransplantatie de enige kans op een blijvende genezing voor CML patiënten. Sinds kort wordt bij de behandeling van CML frequent gebruik gemaakt van Gleevec® (imatinib mesylate), dat selectief de BCR-ABL kinase activiteit inhibeert. Vooral nieuwe gediagnostiseerde CML patiënten in chronische fase (dit is de eerste fase van de ziekte) vertonen een zeer goede cytogenetische en moleculaire respons op deze therapie. In Hoofdstuk 3.4 toonden wij aan dat Gleevec® ook bij patiënten in een vergevorderd stadium van de ziekte na een

niet-verwante beenmergtransplantatie, een blijvende moleculaire remissie kan induceren.

De prognose van AML bij jonge patiënten (≤60 jaar) is de laatste decennia significant verbeterd: veelal bereiken 75-80% van de patiënten een complete remissie en is er een 5-jaarsoverleving van 30-40%. Dit is in scherp contrast met oudere AML patiënten (>60 jaar) waarvoor slechts geringe vooruitgang werd geboekt: complete remissies in 50-55% van de patiënten en een 5-jaarsoverleving van ongeveer 10%. Een gedeeltelijke verklaring is dat ouderen de intensieve chemotherapie niet of moeilijk verdragen. Sinds kort zijn er nieuwe behandelingsmethodes ter beschikking zoals immunotherapie met het gebruik van antistoffen die selectief gericht zijn tegen bepaalde antigenen op de leukemiecellen. Een voorbeeld hiervan is Mylotarg® (gemtuzumab ozogamicine), een monoklonale antistof die gekoppeld is aan het antitumorale calicheamicine en die gericht is tegen het CD33-antigeen, dat aanwezig is op >85% van de AML-cellen. Dertig procent van de oudere AML patiënten met een recidief bereiken na 2 intraveneuze toedieningen van Mylotarg® (dosis: 9 mg/m^2) opnieuw een remissie. In Hoofdstuk 3.5 toonden we aan dat na toediening van deze Mylotarg® doses de CD33-saturatie in het beenmerg veelal beperkt is (40-90%) -dit in tegenstelling tot saturaties van >90% in het perifeer bloed- en dat deze lagere CD33-saturaties *in vitro* leiden tot een minder efficiënte celdoding. Onze experimenten suggereerden dat de mate van CD33-saturatie in het beenmerg wordt bepaald, 1) door het totaal aantal CD33 antigenen in het perifeer bloed (hoge aantallen antigenen in het perifeer bloed binden hoge doses Mylotarg® waardoor er minder product beschikbaar blijft om door te dringen naar het beenmerg), en 2) door het totaal aantal CD33 antigenen op de cellen in het beenmerg zelf. Deze bevindingen impliceren dat voor een efficiënte en optimale doding van de leukemiecellen in het beenmerg hogere of frequentere Mylotarg® doses moet worden toegediend of dat de hoeveelheid CD33 antigenen eerst moet worden verminderd voordat Mylotarg® wordt toegediend bij voorkeur door het ontwerpen van therapieprotocollen, waarin chemotherapeutica en Mylotarg® worden gecombineerd.

Als besluit kunnen we stellen dat de resultaten beschreven in dit proefschrift, 1) bijdragen tot de verdere identificatie van PCR-detecteerbare MRD targets in AML en CML, 2) aantonen dat een perifeer bloedstaal een beenmerg kan vervangen voor PCR-MRD analyses in bepaalde AML-subtypes, 3) illustreren dat MRD detectie met PCR en flowcytometrische immunofenotypering klinisch belangrijk is, en 4) aantonen dat de evaluatie van nieuwe (antistof)therapieën essentieel is om optimale behandelings- en doseringsschema's te bepalen.

MRD-gebaseerde behandelingsinterventies worden reeds toegepast bij bepaalde AML subtypes (bv. AML-M3) en bij getransplanteerde CML patiënten met zeer gunstige resultaten. Verder onderzoek is nodig om na te gaan of MRD-gebaseerde behandelingsinterventies (zowel intensivering van de therapie als reductie ervan) ook bij andere AML-subtypes de kans op genezing kunnen

verhogen. Verder onderzoek naar de identificatie van nieuwe MRD targets maar vooral onderzoek naar nieuwe therapeutische mogelijkheden zullen zeker bijdragen tot hogere genezingskansen voor patiënten met myeloïde leukemie.

DANKWOORD

In januari 1999 begon voor mij ‘mijn Rotterdamse avontuur’. Met klamme handen zette ik mijn eerste stappen in de ‘wereld van de research’. Vandaag, 22 maart 2006, kan iedereen de resultaten van dit erg boeiende en succesvolle ‘avontuur’ lezen in dit boekje.

Mensen die me kennen, weten dat ik niet de grootste prater ben; een uitgebreid dankwoord moet je hier dan ook niet verwachten. Echter, enkelen -zonder wie het allemaal niet gelukt zou zijn- wil ik toch even speciaal vermelden.

Vooreerst prof. dr. J.J.M. van Dongen, mijn Rotterdamse promotor. Jacques, zonder jouw enthousiasme en niet-aflatende wetenschappelijk steun gedurende de voorbije jaren, jouw stimulerende en soms toch licht confronterende gesprekken, zou ik het ‘Rotterdamse avontuur’ niet tot een goed einde hebben gebracht. Mijn welgemeende dank!!

Prof. dr. P. Vandenberghe, mijn Leuvense promotor. Peter, hoewel jij pas in een latere fase mee in mijn onderzoeksproject stapte, kon ik voor de laatste etappes toch steeds op het nodige advies en jouw hulp rekenen. Dank je wel!

Dr. P. Vandekerckhove, mijn co-promotor. Philippe, onder jouw supervisie kwam ik voor het eerst in contact met de hemato-oncologie. Jij gaf me het initiële en nodige duwtje om de uitdaging aan te vatten. Bedankt!

Dr. V.H.J. van der Velden; beste Vincent, bij jou kon ik steeds terecht voor advies, suggesties en het doorlezen van manuscripten. Zeker toen ik terug in Leuven zat, was jij mijn meest directe aanspreek- en contactpersoon op de afdeling. Een oprechte ‘merci’!

De overige leden van de commissie; prof. dr. R. Pieters, prof. dr. B. Löwenberg en prof. dr. G. Verhoef dank ik voor hun inspanning om de teksten op korte tijd door te lezen en te beoordelen. Ook dank ik prof. dr. H. Hooijkaas en prof. dr. G.J. Ossenkoppele voor hun toezegging om plaats te nemen in de grote commissie.

Tevens wil ik beide afdelingshoofden, prof. dr. N. Blanckaert en prof. dr. R. Benner, bedanken voor de mogelijkheden die ik kreeg om me verder te ontplooien in Leuven en Rotterdam.

Speciale dank ook aan alle MLT’s en analisten; PCR’s inzetten, gels runnen, … jullie hebben het mij allemaal geleerd en nog vaker voor mij uitgevoerd! Ook een bloemetje voor de secretaresses Wendy, Marcia, Daniëlle en Erna; zonder jullie hulp zat ik waarschijnlijk nog te zwoegen aan de lay-out en de referenties. Tevens ook een woord van dank aan Marieke Comans en Tar van Os voor hun hulp bij het opmaken van de figuren en de cover. Dank ook aan collega’s, familie en vrienden, en vooral…. BEDANKT MAMA EN PAPA voor jullie onvoorwaardelijke steun en blijvende belangstelling!!!

CURRICULUM VITAE

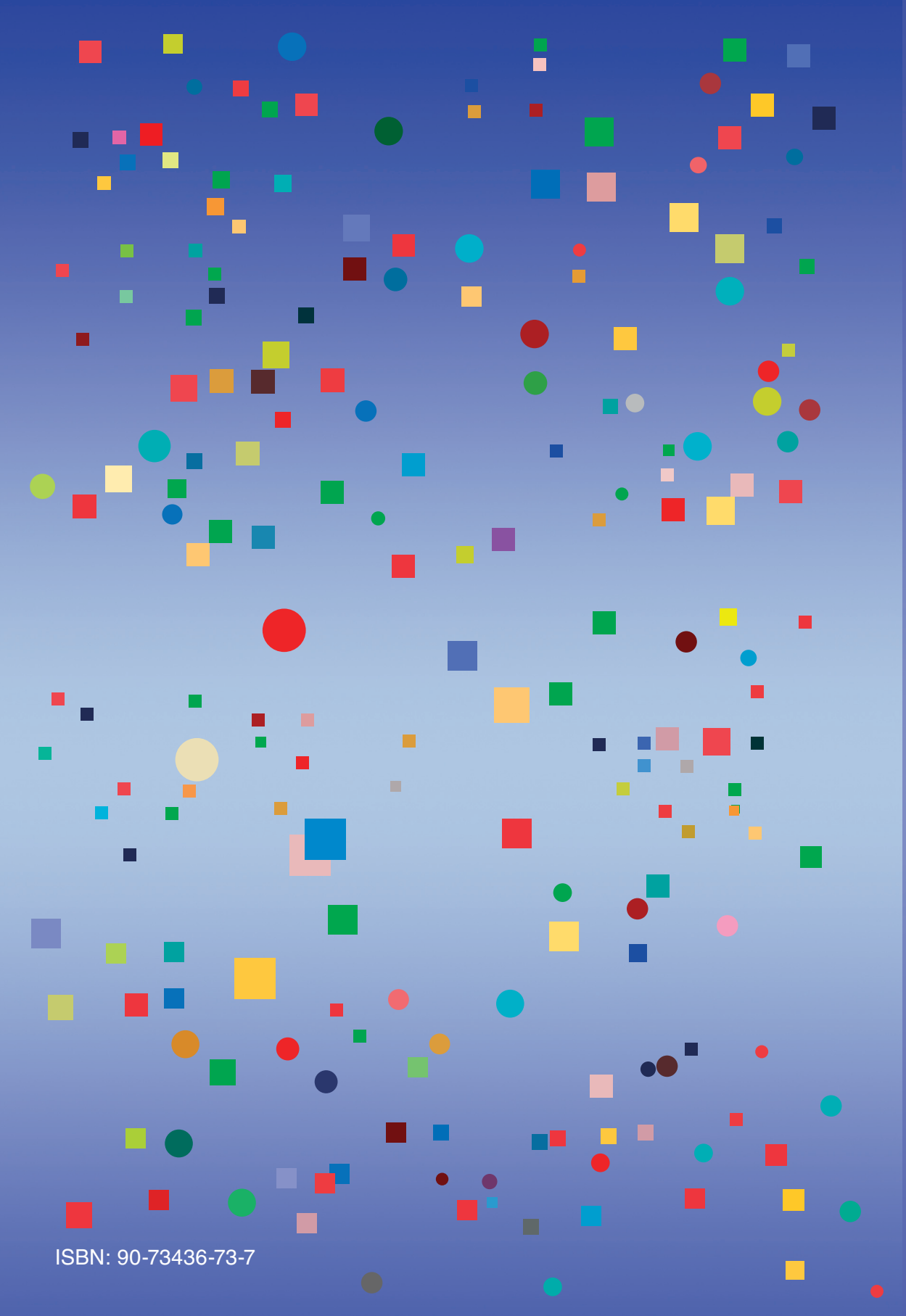
Nancy Boeckx

26 maart 1970	Geboren te Mortsel
1982 - 1985	Secundair onderwijs: Latijn-Grieks *(Kardinaal van Roey Instituut, Vorselaar)*
1985 - 1988	Secundair onderwijs: Latijn-Wetenschappen *(Kardinaal van Roey Instituut, Vorselaar)*
1988 - 1995	Opleiding tot Arts in de Genees-, Heel- en Verloskunde *(Katholieke Universiteit Leuven)*, Promotie juli 1995
1995 - 2001	Opleiding tot Geneesheer-Specialist in de Klinische Biologie *(Katholieke Universiteit Leuven)*, Promotie juli 2001
Augustus 2001 - juli 2002	Resident Klinisch Bioloog, afdeling Laboratoriumgeneeskunde, Hematologie *(Universitair Ziekenhuis Gasthuisberg, Leuven)*
2001 - heden	Gastlector, Katholieke Hogeschool Leuven, Departement Rega
Augustus 2002 - juli 2004	Supervisor Klinisch Bioloog, afdeling Laboratoriumgeneeskunde, Hematologie *(Universitair Ziekenhuis Gasthuisberg, Leuven)*
Augustus 2004 - heden	Adjunct-Kliniekhoofd Klinisch Bioloog, afdeling Laboratoriumgeneeskunde, Hematologie *(Universitair Ziekenhuis Gasthuisberg, Leuven)*
1999 - 2005	Promotieonderzoek “Molecular and flow cytometric diagnostics for evaluation of therapy efficacy in myeloid leukemias” onder leiding van prof. dr. J.J.M. van Dongen, prof. dr. P. Vandenberghe en dr. P. Vandekerckhove (Afdeling Immunologie, Erasmus MC, Rotterdam en Afdeling Laboratoriumgeneeskunde, Hematologie, Universitair Ziekenhuis Gasthuisberg, Leuven)

PUBLICATIONS

1. L Ide, A Dekoninck, E Verburgh, W Goossens, C Brusselmans, **N Boeckx**, MP Emonds, P Vandekerckhove. Persistent polyclonal B-cell lymphocytosis. *Acta Clin Belg* 2002; **57**: 31-33.
2. **N Boeckx**, M Willemse, T Szscepanski, TW Langerak, VHJ van der Velden, P Vandekerckhove, JJM van Dongen. Fusion gene and Ig/TCR gene rearrangements are complementary but infrequent targets for PCR-based detection of minimal residual disease in acute myeloid leukaemia. *Leukemia* 2002; **16**: 368-375.
3. P Vandenberghe, **N Boeckx**, E Ronsyn, M Boogaerts, G Verhoef, A Hagemeijer. Imatinib mesylate induces durable complete remission of advanced CML persisting after allogeneic bone marrow transplantation. *Leukemia* 2003; **17**: 458-460.
4. VHJ van der Velden, PG Hoogeveen, **N Boeckx**, MJ Willemse, E Delabesse, V Asnafi, E MacIntyre, N Pallisgaard, P Hokland, LS Mikkelsen, JM Cayela, O Spinelli, JJM van Dongen. Intrachromosomal microdeletion on 1p32 with the SIL-TAL1 fusion gene transcript. Published as a part of: Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia – A Europe Against Cancer Program. J. Gabert *et al*. *Leukemia* 2003; **17**: 1-40.
5. **N Boeckx**, A Uyttebroeck, AW Langerak, C Brusselmans, W Goossens, X Bossuyt. Clonal proliferation of T-cell large granular lymphocytes. *Pediatr Blood Cancer* 2004; **42**: 275-277.
6. VHJ van der Velden, **N Boeckx**, M Gonzales, M Malec, G Barbany, T Lion, G Saglio, N Pallisgaard, E Beillard, W Bi, WCJ Hop, PG Hoogeveen, JJM van Dongen, J Gabert on behalf of the Europe Against Cancer Program. Differential stability of control gene and fusion gene transcripts over time may hamper accurate detection of minimal residual disease – A Europe Against Cancer Program. *Leukemia* 2004; **18**: 884-886.
7. VHJ van der Velden, **N Boeckx**, I Jedema, JG te Marvelde, PG Hoogeveen, M Boogaerts, JJM van Dongen. High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg®) treatment in acute myeloid leukemia patients. *Leukemia* 2004; **18**: 983-988.
8. **N Boeckx,** VHJ van der Velden, M Boogaerts, A Hagemeijer, P Vandenberghe, JJM van Dongen. An inv(16)(p13q22) positive acute myeloid leukaemia relapsing as acute precursor B-cell lymphoblastic leukaemia. *Haematologica* 2004; **89**: ECR28.
9. VHJ van der Velden, **N Boeckx**, ER van Wering, JJM van Dongen. Detection of minimal residual disease in acute leukemia. *J Biol Regul Homeos Agents* 2004; **18**: 146-154.

10. C Graux, J Cools, C Melotte, H Quentmeier, A Ferrando, R Levine, JR Vermeesch, M Stul, B Dutta, **N Boeckx**, A Bosly, P Heimann, A Uyttebroeck, N Mentens, R Somers, RAF MacLeod, HG Drexler, AT Look, DG Gilliland, L Michaux, P Vandenberghe, I Wlodarska, P Marynen, A Hagemeijer. Fusion of NUP214 to ABL1 on amplified episomes in T-cell acute lymphoblastic leukaemia. *Nat Genet* 2004; **36**: 1084-1089.
11. **N Boeckx,** MW Jansen, C Haskovec, P Vandenberghe, VHJ van der Velden, JJM van Dongen. Identification of e19a2 BCR-ABL fusions (µ-BCR breakpoints) at the DNA level by ligation-mediated PCR. *Leukemia* 2005; **19**: 1292-1295.
12. **N Boeckx**, J De Roover, VHJ van der Velden, J Maertens, A Uyttebroeck, P Vandenberghe, JJM van Dongen. Quantification of *CBFB-MYH11* fusion gene levels in paired peripheral blood and bone marrow samples by real-time PCR. *Leukemia* 2005; **19**: 1988-1990.
13. VHJ van der Velden, JG te Marvelde, PG Hoogeveen, **N Boeckx**, JJM van Dongen. Evaluatie van antistof-gemedieerde therapie bij hematopoietische maligniteiten. In: Nieuwe ontwikkelingen in de Medische Immunologie. Editors: H. Hooijkaas, JJM. van Dongen. Dept. of Immunology, Erasmus MC, Rotterdam, The Netherlands: 2005; 141-151.
14. J Cloos, BF Goemans, C Hess, JW van Oostveen, Q Waisfisz, S Corthals, D de Lange, **N Boeckx**, K Hählen, D Reinhardt, U Creutzig, GJ Schuurhuis, CM Zwaan, GJL Kaspers. Presence of activating mutations in the *FLT*-3 gene is related to time to relapse as measured in paired initial and relapse AML samples. *To be submitted*.
15. A Uyttebroeck, A Hagemeijer, **N Boeckx**, M Renard, I Wlodarska, P Vandenberghe, C De Wolf-Peeters. Is there a difference in childhood T-cell acute lymphoblastic leukaemia and T-cell lymphoblastic lymphoma? *To be submitted*.



ISBN: 90-73436-73-7