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Detektion minimaler Resterkrankung bei der Akuten Myeloischen Leukämie mit t(8;21) Translokation
Detection of minimal residual disease in Acute Myeloid Leukemia with t(8;21) translocation

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1. INTRODUCTION

1.1 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a group of heterogeneous hematopoietic neoplasms characterized by the clonal proliferation of myeloid precursors, as a result of the loss of ability to respond to normal control mechanisms of cell proliferation and differentiation into more mature cells. The annual incidence of AML is approximately 4 cases per 100,000 population (Deschler and Lubbert, 2006). Although the disease occurs at a young age, the median age of diagnosis is 70 years (Estey and Dohner, 2006, Juliusson et al., 2009).

1.1.1 Etiology and Pathophysiology of AML

Haematopoiesis includes all the processes of proliferation and differentiation of the progenitor hematopoietic stem cells into more mature cells, myelocytes, lymphocytes, and megakaryocytes. Creating and maintaining appropriate conditions in the microenvironment of the bone marrow (BM), is of great importance in order to preserve an effective haematopoiesis (Colmone et al., 2008). In AML, the differentiation of myeloid progenitor cells is impaired and the apoptotic mechanisms are inhibited. This arrest in maturation results in uncontrolled proliferation and accumulation of myeloid immature cells (blasts) in the BM and in the peripheral blood (PB), as well as in the infiltration of other tissues, referred to as extramedullary disease (Ohanian et al., 2013). Often this leads to hematopoietic insufficiency (anemia, neutropenia, thrombocytopenia) with or without leucocytosis, due to BM failure.

AML is clinically and biologically, a heterogeneous group of diseases, as a result of the large number of genetic and epigenetic events (Gutierrez and Romero-Oliva, 2013, Popp and Bohlander, 2010). A great deal of evidence suggests that proto-oncogenes and other growth-promoting genes such as those encoding for cytokines or their receptors, play an important role in leukemogenesis. In this evolutionary process genetic changes such as chromosomal aberrations or deletions may alter the regulation and the function of the proto-oncogenes and of the growth-promoting genes (Irons and Stillman, 1996). Intensive research activity has led to the conclusion that translocations observed in leukemias, may take place early in the

process of leukemogenesis since they appear to be stable and balanced within the leukemic clone (Kennedy and Barabe, 2008).

Several risk factors have been associated with the development of AML. These include age, genetic disorders, as well as exposure to viruses, to ionizing radiation, to chemicals and to other occupational hazards (Sandler and Ross, 1997). Previous exposure to cytotoxic therapy with alkylating agents and topoisomerase II inhibitors (Momota et al., 2013, Baehring and Marks, 2012), has been reported to increase the incidence of leukemia, and has been related to specific cytogenic changes: deletions or loss of 7q or 5q as well as 11q23 chromosomal abnormalities respectively (Tang et al., 2012, Ezoe, 2012). Additionally, exposure to benzene (Irons et al., 2013) and cigarette smoking are also possible etiological factors (Sandler and Collman, 1987, Pogoda et al., 2002, Smith et al., 2011). Despite these associations, at the present time only 1-2% of the diagnosed leukemias can be attributed to exposure to these agents (Fernberg et al., 2007).

1.1.2 Classification of AML

In 1976, a new morphologic classification for acute leukemias was proposed by a working committee of French, American and British haematologists.

Since its introduction this system known as FAB (French-American-British) classification has been widely accepted internationally. It is based on Romanovsky-stained blast morphology and on cytochemical stains (Bennett et al., 1976). At that time FAB classification required the presence of 30% blasts in bone marrow, as a criterion of diagnosis. It divides the AML into eight subtypes depending on the degree of maturation of the particular myeloid lineage involved. The distinction is based on the morphologic appearance of the blasts and their reactivity with the histochemical stains. Additionally, immunologic methods have been incorporated into the diagnostic criteria for some FAB subgroups (Lowenberg et al., 1999) (Table 1).

Table 1: FAB classification of AML

FAB SUBTYPE	COMMON NAME (% OF CASES)	RESULTS OF STAINING			ASSOCIATED TRANSLOCATIONS AND REARRANGEMENTS (% OF CASES)	GENES INVOLVED
		MYELO-PEROXI-DASE	SUDAN BLACK	NON SPECIFIC ESTERASE		
M0	Acute myeloblastic leukemia with minimal differentiation (3%)	-	-	-*	Inv(3q26) and t(3;3)(1%)	*EVI1*
M1	Acute myeloblastic leukemia without maturation (15-20%)	+	+	-		
M2	Acute myeloblastic leukemia with maturation (25-30%)	+	+	-	t(8;21)(40%), t(6;9)(1%)	*AML1-ETO, DEK-CAN*
M3	Acute promyelocytic (5-10%)	+	+	-	t(15;17)(98%), t(11;17)(1%), t(5;17)(1%)	*PML-RARα, PLZF-RARα, NPM1-RARα*
M4	Acute myelomonocytic leukemia (20%)	+	+	+	11q23(20%), inv(3q26) and t(3;3)(3%), t(6;9)(1%)	*MLL, DEK-CAN, EVI1*
M4E0	Acute myelomonocytic leukemia with abnormal eosinophils (5-10%)	+	+	+	Inv(16), t(16;16)(80%)	*CBFβ-MYH11*
M5	Acute monocytic leukemia (2-9%)	-	-	+	11q23(20%), t(8;16)(2%)	*MLL, MOZ-CBP*
M6	Erythroleukemia (3-5%)	+	+	-		
M7	Acute megakaryocytic leukemia (3-12%)	-	-	+†	t(1;22)(5%)	*Unknown*

*Cells are positive for myeloid antigen (e.g., CD13 and CD33).
†Cells are positive for a-naphthylacetate and platelet glycoprotein IIb/IIIa or factor VIII–related antigen and negative for naphthylbutyrate
(Adapted from NEJM (Lowenberg et al., 1999))

Over the years, many large clinical studies highlighted the value of cytogenetic abnormalities in acute leukemias, thus requiring the revision of FAB classification. The importance of genetic events to diagnose and treat acute leukemia became widely accepted and a new classification was proposed from World Health Organization (WHO), in 2001 (Table 2). In this late classification acute leukemias are divided into 4 major groups. The genetic aberrations play a key role and the

percentage of blasts required for diagnosis of AML is lowered from 30% to 20% in PB and/or the BM aspirate. Exceptions include AML with t(8;21), inv(16) or t(15;17), in which the diagnosis of AML is made in spite a blast percentage in the BM<20%. This classification includes the genetic aberrations and immunophenotyping as major defining features in addition to morphology (Vardiman et al., 2002).

Table 2: WHO classification of AML (WHO 2001)

WHO classification of AML
Acute myeloid leukemia with recurrent genetic abnormalities • Acute myeloid leukemia with t(8;21)(q22;q22),(*AML1/ETO*) • Acute myeloid leukemia with abnormal bone marrow eosinophils inv(16)(p13q22) or t(16;16)(p13;q22),*(CBFβ/MYH11)* • Acute promyelocytic leukemia with t(15;17)(q22;q12)(*PML/RARa*) and variants • Acute myeloid leukemia with 11q23 (*MLL*) abnormalities
Acute myeloid leukemia with multilineage dysplasia • Following MDS or MDS/MPD • Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of cells in 2 or more myeloid lineages
Acute myeloid leukemia and myelodysplastic syndromes, therapy related • Alkylating agent/radiation related-type • Topoisomerase type II inhibitor-related (some may be lymphoid) • Others
Acute myeloid leukemia not otherwise categorised Classify as: • Acute myeloid leukemia minimally differentiated • Acute myeloid leukemia without maturation • Acute myeloid leukemia with maturation • Acute myelomonocytic leukemia • Acute monoblastic/acute monocytic leukemia • Acute erythroid leukemia (erythroid/myeloid and pure erythroleukemia) • Acute megakaryoblastic leukemia • Acute basophilic leukemia • Acute panmyelosis with myelofibrosis • Myeloid sarcoma

(Adapted from Blood journal, (Vardiman et al., 2002))

Since 2001, there has been considerable progress in understanding the biology of the disease. The discovery of many molecular abnormalities in myeloid neoplasms and the need for a common language between clinicians and laboratory investigators

has led to the publication of another revision of the classification of hematologic neoplasms (Vardiman et al., 2009, Dohner et al., 2010). It has been published as part of the 4th edition (Vardiman et al., 2009) of the WHO, where new categories as well as new provisional entities have been incorporated (Table 3).

Table 3: Acute myeloid leukemia and related precursor neoplasms, and acute leukemias of ambiguous lineage (WHO 2008)

Categories
Acute myeloid leukemia with recurrent genetic abnormalities
• AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
• AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
• Acute promyelocytic leukemia with t(15;17)(q22;q12); *PML/RARA*
• AML with t(9;11)(p22;q23) *MLLT3-MLL*
• AML with t(6;9)(p23;q34); *DEK-NUP214*
• AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
• AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
• *Provisional entity: AML with mutated NPM1*
• *Provisional entity: AML with mutated CEBPA*
Acute myeloid leukemia with myelodysplasia-related changes
Therapy related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified (NOS)
• Acute myeloid leukemia with minimal differentiation
• Acute myeloid leukemia without maturation
• Acute myeloid leukemia with maturation
• Acute myelomonocytic leukemia
• Acute monoblastic/monocytic leukemia
• Acute erythroid leukemia Pure erythroid leukemia Erythroleukemia, erythroid/myeloid
• Acute megakaryoblastic leukemia
• Acute basophilic leukemia
• Acute panmyelosis with myelofibrosis (syn.: acute myelofibrosis; acute myelosclerosis)
Myeloid sarcoma (syn.: extramedullary myeloid tumor; granulocytic sarcoma; chloroma)
Myeloid proliferations related to Down syndrome
• Transient abnormal myelopoiesis (syn.: transient myeloproliferative disorder)
• Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

Table 3: Acute myeloid leukemia and related precursor neoplasms, and acute leukemias of ambiguous lineage (WHO 2008) (continued)

Acute leukemias of ambiguous lineage
• Acute undifferentiated leukemia
• Mixed phenotype acute leukemia with t(9;22)(q34;q11.2);*BCR-ABL1*
• Mixed phenotype acute leukemia with t(v;11q23); *MLL* rearranged
• Mixed phenotype acute leukemia, B/myeloid, NOS
• Mixed phenotype acute leukemia with, T/myeloid NOS
• *Provisional entity: Natural killer (NK)-cell lymphoblastic leukemia/lymphoma*

(Adapted from Blood journal (Dohner et al., 2010))

1.2 Two-hit model of AML

The pathogenesis of AML requires a series of genetic events (Jacobs, 1991, Dohner and Dohner, 2008). The specific mutational events required for this progression are not currently well defined. Based on experimental data from mouse bone marrow transplantation models, G.Gililland (Gilliland, 2001) proposed the "two-hit model" of leukemogenesis. According to this hypothesis, AML is the consequence of collaboration of at least two classes of mutations (Fig 1).

- class I mutations: the first type of genetic lesion involves mutations that disturb the signal transduction pathways, favouring the proliferation and/or the survival of the cells. Already recognised mutations belonging to this category are:
  - **Mutations leading to continuous activation of FLT3 receptor**
    FLT3 is a transmembrane receptor and belongs in PDGFR subfamily (class III) of tyrosine kinase receptors which also include PDGFRA, PDGFRB, FMS and KIT (Small, 2006, Levis and Small, 2003). These receptors present the following common structure: a) 5 extracellular immunoglobulin domains, b) a transmembrane domain, c) a juxtamembrane domain and d) an intracellular tyrosine kinase domain (TK) (Frohling et al., 2002). FLT3 receptor is expressed in progenitor stem cells and plays a major role in survival, proliferation and differentiation through signal transduction pathways like RAS/Raf/Mek/Erk or STAT (Small, 2006). In AML two types of mutations have been recognized:

a) mutations of the internal tandem duplication (ITD)(*FLT3*-ITD) seen in 23% of patients with AML (Small, 2006).
b) point mutations which usually involve codon 835 (*FLT3*-Asp835) of the kinase domain and is found in 8-12% of the AML patients (Small, 2006).

The presence of *FLT3* mutations is of major clinical significance. Patients with normal karyotype (NK) harboring the mutation *FLT3*-ITD have an inferior outcome (Schlenk et al., 2008, Kottaridis et al., 2003, Gale et al., 2008).

- **Mutations in the *RAS* gene family**

  RAS gene encodes a G protein, which plays a major role in signal transduction, cell proliferation and malignant transformation.

  Two types of mutations are recognized:

  a) *NRAS* mutations are found in 9-14% of cytogenetically normal AML adult patients (Dohner, 2007), in about 40% of patients with core binding factor (CBF) AML and in 25% of patients with inv(3) AML (Dohner and Dohner, 2008).
  b) *KRAS* mutations are found in 5-17% of CBF AML (Dohner and Dohner, 2008).

- ***JAK2*V617F mutation**

  *JAK2*V617F mutation is responsible for the increase activity of JAK/STAT signaling pathway which will result in the uncontrolled cell proliferation and survival (Kralovics et al., 2005, Schnittger et al., 2007a) (the mechanism is analyzed in paragraph 1.4, page 17).

- ***KIT* mutations**

  C-KIT is a receptor of tyrosine kinase (RTK) with a central role in hematopoiesis and in leukemogenesis (Malaise et al., 2009, Becker et al., 2008). Mutations in the tyrosine kinase domain at codon 816 (*KIT*-D816) are present in about one-third of CBF leukemias (Zheng et al., 2009, Cairoli et al., 2006, Paschka and Dohner, 2013).

  Recent studies indicate the adverse effect of the mutation, in the outcome of patients with t(8;21) (Cairoli et al., 2006, Schnittger et al., 2006b). *KIT* mutations have negative impact on survival and event free survival in these patients (Schnittger et al., 2006b), while the impact of the mutation in patients with inv(16) is not clear (Paschka and Dohner, 2013, Kim et al., 2013).

- Class II mutations: according to the model that is proposed from Gililland, the second type of genetic lesion involves mutations affecting transcription factors and/or the transcriptional co-activation complex. This will lead to the impairment of the differentiation process (Dohner and Dohner, 2008). Known mutations in this category are:
  - **Mutations in *CEBPA***

    The CCAAT enhancer binding protein alpha (*CEBPA)* gene encodes a member of the basic region leucine zipper (bZIP) transcription factors important for the differentiation of myeloid cells (Nerlov, 2004). The frequency of *CEBPA* mutations in NK-AML is 10-18% (Dufour et al., 2010, Dohner and Dohner, 2008) and the presence of biallelic mutation has been associated with a better overall survival (OS) (Dufour et al., 2010, Dufour et al., 2012, Taskesen et al., 2011).
  - ***NPM1* mutations**

    Mutations occurring in exon 12 of the nucleophosmin 1 gene (*NPM1*) are the most frequent genetic abnormalities in patients with *de novo* AML-NK (60%) (Falini et al., 2005, Falini et al., 2007a). Falini *et al* showed that the most common *NPM1* mutation is the duplication of TCTG tetranucleotide named mutation A (Falini et al., 2007b). *NPM1* is located in the nucleolus and shuttles continuously between nucleus and cytoplasm. It is associated with the nucleolar ribonucleoprotein (ribosome biogenesis) (Falini et al., 2007b, Sportoletti, 2011).

    Patients with *NPM1* mutations without *FLT3*-ITD have higher remission rates and favorable relapse-free survival (RFS) and OS (Dohner et al., 2010, Estey, 2013).
  - **Fusion genes resulting from translocations t(8;21), inv(16)/t(16;16) and t(15;17)**

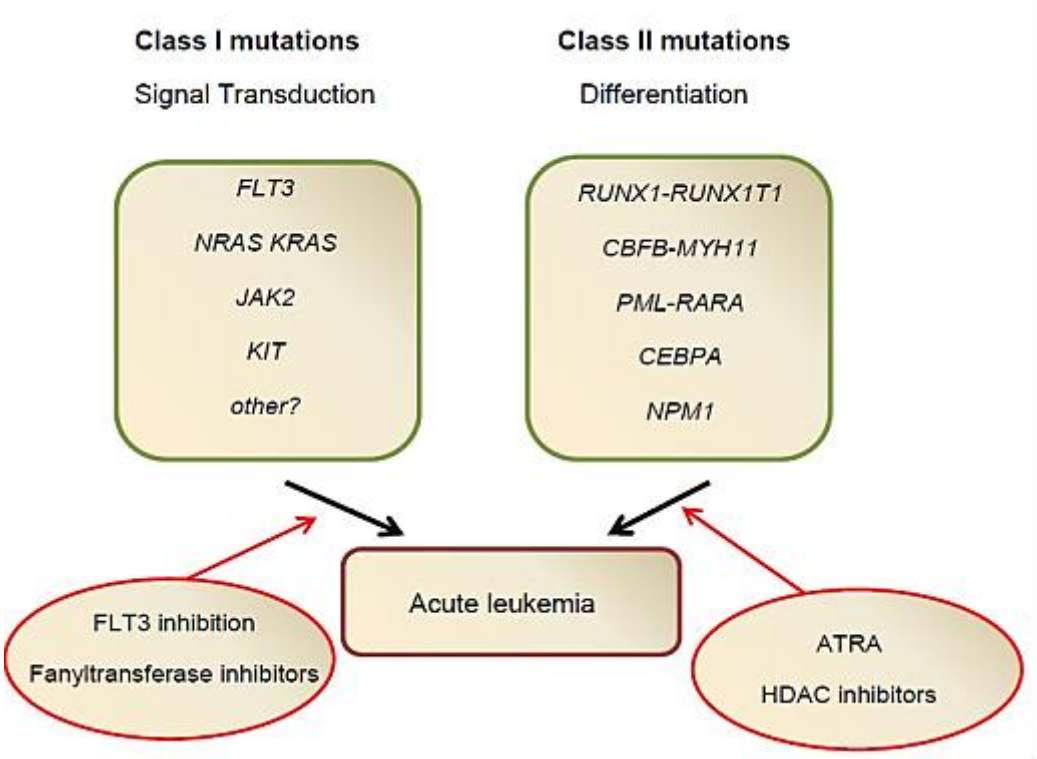
    Leukemias with t(8;21)(q22;q22) (described in paragraph 1.3) and inv16(p13;q22)/t(16;16)(p13;q22), are known as core binding factor (CBF) leukemias and belong to the favorable cytogenetic risk group (Dohner and Dohner, 2008, Yin et al., 2012).

    The reciprocal translocation t(15;17)(q22;q21), characterizes acute promyelocytic leukemia (APL) and has as result the formation of *PML-RARA* hybrid gene. *PML-RARA* is detected in 98% of APL cases.

    APL is a unique entity and the most curable myeloid leukemia (Lo-Coco et al., 2008). The early detection and molecular monitoring of

PML-RARA fusion transcripts, is of crucial importance, since molecular relapse predicts hematological relapse (Grimwade and Lo Coco, 2002).

Figure 1: **The two hit model of AML**



1.3 Acute myeloid leukemia with translocation t(8;21)

1.3.1 Clinical features associated with t(8;21) leukemia

AML cases with t(8;21) constitute about 5% of all AML cases (Vardiman et al., 2009). According to the last WHO classification (Vardiman et al., 2009), the translocation can be observed in about 10% of AML M2 FAB subtype, and in about 6% of AML M1 FAB subtype (Peterson et al., 2007). It is one of the most important clinical subtypes in AML (Rowley, 2000, Ferrara and Del Vecchio, 2002).

From a clinical point of view, AML with t(8;21) tends to occur in patients of a younger age (mostly <60 years) and is usually associated with a high remission rate and a prolonged disease-free survival (DFS) and OS in patients treated with standard induction and consolidation chemotherapy (Bloomfield et al., 1998, Grimwade et al., 1998, Cho et al., 2003, Dohner et al., 2010). The morphological features include the presence of blasts with abundant basophilic cytoplasm containing in most of the cases, numerous azurophilic granules and blasts with characteristic Auer rods (Arber et al., 2003)..

In addition, the leukemic blasts frequently have a distinct immunophenotype, characterized by the expression of myeloid markers (CD13, CD33, MPO), and also by the co-expression of the lymphoid marker CD19. CD34 is characteristically present, and CD56 is often expressed (Arber et al., 1997, Zheng et al., 2008, De et al., 2007).

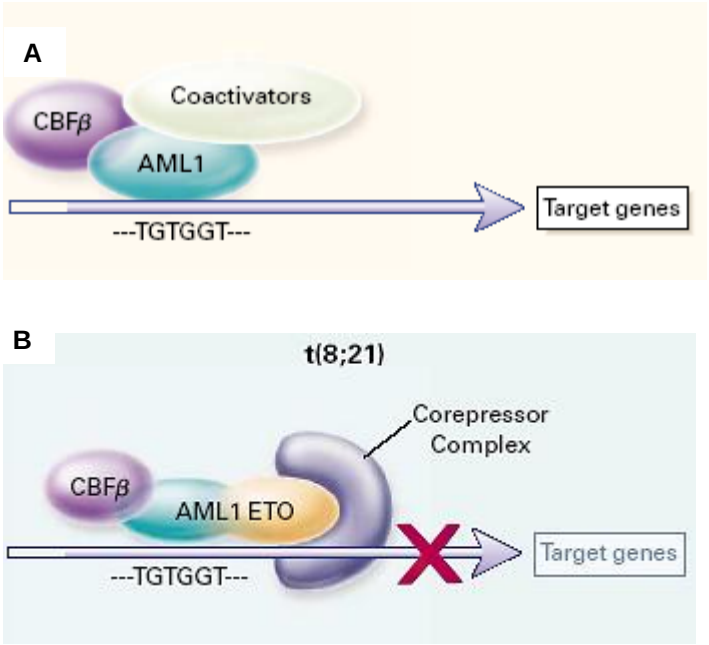
1.3.2 Biology of RUNX1-RUNX1T1 chimeric transcription factor

More than 3 decades age the specific morphology associated with the t(8;21) translocation was defined and scientists came to an understanding of the genes involved (Rowley, 2000).

The translocation t(8;21)(q22;q22) involves the *AML1* gene (also known as *RUNX1)*, located on chromosome 21, and the Eight-Twenty-One (*ETO, MTG8* or *RUNX1T1*) gene, located on chromosome 8 (Chang et al., 1993, Erickson et al., 1992, Reikvam et al., 2011). According to the HGNC nomenclature *RUNX1-RUNX1T1* is now the official name, although *AML1-ETO* is still common. It is responsible for the fusion of the N-terminal DNA-binding domain of the *RUNX1* gene with almost the entire *RUNX1T1* gene (including the region coding for the 4 C-terminal domains) (Erickson et al., 1992). RUNX1 is a key transcription factor for various hematopoietic specific genes. Less is known for the function of *RUNX1T1*, which is believed to be a nuclear co-repressor gene (Reikvam et al., 2011, Plevin et al., 2006).

RUNX1 or CBFA, is the DNA-binding subunit of the core-binding transcription factor (CBF). CBF is composed of two subunits CBFA and CBFB (Leroy et al., 2002). It binds to the enhancer core sequence TGT/GGT (Figure 2–Panel A), which has been shown to be important in the transcriptional regulation of a number of viral, and cellular genes (Wang et al., 1993). The DNA binding activity of RUNX1 is mediated through a central 118 amino acid domain that is homologous to the Drosophlila pair-rule protein Runt, hence it is designated as the Runt homology domain (RHD) (Crute et al., 1996, Daga et al., 1996). This binding affinity is increased through heterodimerization of the RDH with a second non-DNA-binding subunit CBFB (Wang et al., 1993). RUNX1 has been shown to function as a transcription activator and it is of critical importance since it regulates the expression of the following haematopoietic specific genes: myeloperoxidase, granulocyte-colony-stimulating factor 1 (G-CSF) receptor, subunits of the T-cell antigen receptor, neutrophil elastase and the cytokines interleukin (IL) -3 and macrophage–colony-stimulating factor (M-CSF) receptor (Nuchprayoon et al., 1997, Zhang et al., 1994, Prosser et al., 1992, Shoemaker et al., 1990).

Figure 2: The RUNX1 Transcription Factor



Panel A: normal cells. Panel B: AML cells with t(8;21)
(Adapted from NEJM (Lowenberg et al., 1999))

RUNX1T1 is the mammalian homologue of the Drosophila gene *nervy* (Feinstein et al., 1995) and contains four evolutionarily conserved domains, the so-called nervy homology regions (NHR) 1-4, which have been shown to interact with co-repressors and histone deacetylases (HDAC) (Amann et al., 2001). RUNX1T1 phosphoprotein is expressed in CD34+ haematopoietic progenitors (Era et al., 1995, Erickson et al., 1996).

In the RUNX1-RUNX1T1 fusion protein the transcriptional activation domains of RUNX1 are replaced by *RUNX1T1* sequences known to interact with nuclear co-repressors like N-CoR, SMRT and HDAC (Downing, 1999) (Fig 2- Panel B). Therefore, RUNX1-RUNX1T1 retains the ability to bind to the core enhancer sequence and to interact with CBFB. However, instead of activating transcription, it functions as a transcriptional repressor, inhibiting the normal transcriptional activity of the wildtype *RUNX1-CBFB*. RUNX1-RUNX1T1 targets the promoters of *RUNX1* target genes and directly represses *RUNX1*-mediated transcriptional activation (Meyers et al., 1995). It also represses *CEBPA* transcriptional activation (Westendorf et al., 1998) and the basal transcription of the multidrug resistance (*MDR*) gene (Lutterbach et al., 1998). Although the majority of data suggests that RUNX1-RUNX1T1 functions as a transcriptional repressor, it has also been found to activate transcription of the *BCL2* promoter (Klampfer et al., 1996).

1.4 JAK-STAT signalling pathway and *JAK2*V617F mutation in Myeloid Disorders

The Janus Kinase (JAK) / signal transducer and activator of transcription (STAT) cascade is an intracellular signalling pathway required for response to many extracellular ligands. It is widely used by members of the cytokine receptor superfamily, including receptors that are important in haematopoiesis (granulocyte colony-stimulating factor, erythropoietin, thrombopoietin, interferons and interleukins) (Yamaoka et al., 2004, Ward et al., 2000).

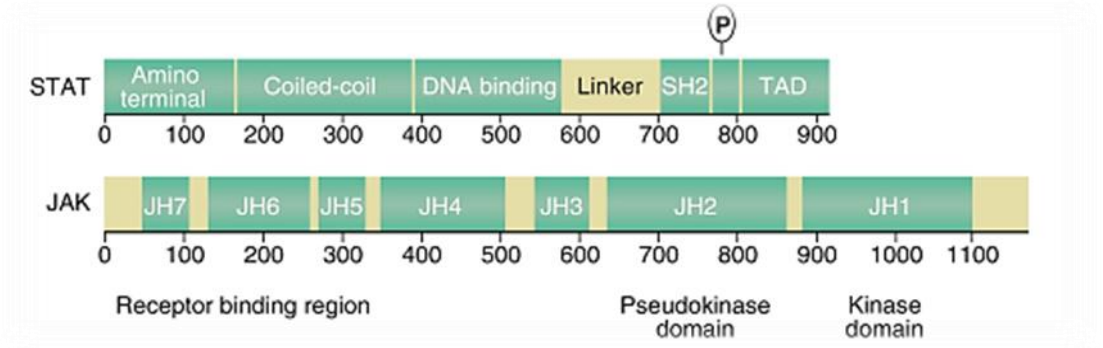
Four cytoplasmic tyrosine kinases (JAK1, JAK2, JAK3 and TYK2) and seven STAT proteins (STAT1 to 6, including STAT5a and STAT5b) have been identified in mammalian cells (Ward et al., 2000).

JAKs consist of seven regions of homology (JH) domains named Janus homology domain 1 to 7 (Becker et al., 1998, Chen et al., 1998, Schindler, 2002) (Fig 3 (Schindler, 2002)). The C-terminal domain (JH1) contains the tyrosine kinase

function and is preceded by a pseudokinase domain (JH2). Its sequence shows high homology to functional kinases, but it does not possess any catalytic activity (Wilks et al., 1991). The N-terminal portion of the JAKs (spanning JH7 to JH3) is important for the receptor association and the non-catalytic activity (Frank et al., 1994).

STATs consist of five domains which include: an amino-terminal domain (NH_2), a coiled-coil domain, the DNA binding domain, a linker domain, an SH2 domain, and a tyrosine kinase domain (P) (Schindler, 2002). In the carboxy-terminus there is a transcriptional activation domain (TAD) which is conserved in function (between homologues), but not in sequence (Becker et al., 1998, Chen et al., 1998) (Figure 3 (Schindler, 2002)).

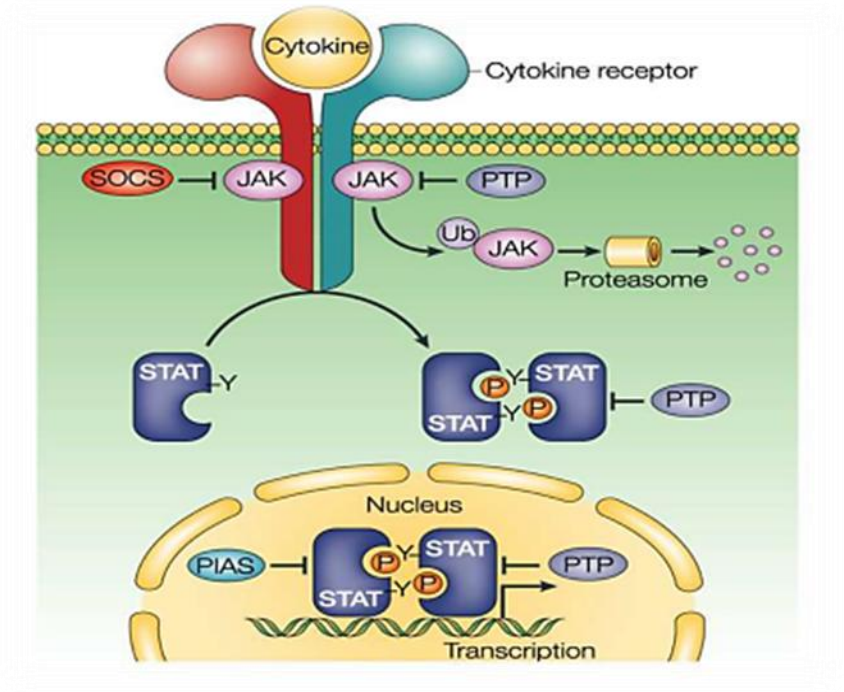
Figure 3: STAT and JAK structure



(adapted from: J Clin Invest (Schindler, 2002))

The JAK-STAT signalling pathway is activated after binding of the specific cytokine with the receptor. This leads to the phosphorylation of specific receptor tyrosine residues (Schindler, 2002). As a result, STAT binds to the phosphorylated receptor and becomes also phosphorylated. After that the activated STAT protein is released from the receptor, it dimerizes and finally is transported into the cell nucleus to activate transcription of target genes (Fig 4 (Shuai and Liu, 2003)). Now JAK-STAT mediated signal transduction is known to regulates many cellular processes through the signalling of cytokines (Schindler, 2002).

Figure 4: JAK2-STAT5 signaling pathway



(Adapted from Nat Rev Immunol. (Shuai and Liu, 2003))

In 2005 a novel somatic point mutation in the autoinhibitory domain of the *JAK2* was described (Baxter et al., 2005, James et al., 2005, Levine et al., 2005, Zhao et al., 2005, Kralovics et al., 2005). The mutation is referred to as V617F. It is the result of the substitution of valine from phenylalanine, at position 617 of the JAK2 protein, within the JH2 pseudokinase domain, which is involved in the inhibition of kinase activity. Loss of JAK2 autoinhibition results in uncontrolled activation of the kinase**,** thus cell proliferation becomes independent of the control of the normal growth factor. The mutation is very common in chronic myeloproliferative neoplasms (MPNs). It is detected in about 95% of patients with polycythemia vera (PV) (Tefferi, 2007) and in 35-50% of patients with essential thrombocythemia (ET), or myelofibrosis with myeloid metaplasia (MMM) (Kralovics et al., 2005, Baxter et al., 2005, Levine et al., 2005, Nelson and Steensma, 2006). The prevalence of *JAK2*V617F seems to be low in myelodysplastic syndrome (MDS) (about 7%) (Steensma et al., 2005) and in atypical mylodysplastic/myeloproliferative disorders (Steensma et al., 2006). In *de novo* AML the incidence of the mutation is approximately 4%-6% (Steensma et al., 2005, Dohner et al., 2006), but it should be mentioned that in about 20-25% of AML patients has been reported increase activity of STAT3 (Steensma et al., 2006).

1.5 Monitoring of minimal residual disease in acute myeloid leukemia

1.5.1 General aspects of minimal residual disease

AML is a heterogeneous disease, as reflected by differences in the morphology of the leukemic blasts and by variations in the clinical picture and therapeutic outcome. Over the past 30 years, remarkable progress was made in understanding the biology of haematological malignancies and consequently new treatment modalities became feasible. Thus, with the contemporary improved risk assessment, chemotherapy, haematopoietic stem cell transplantation (HSCT) and supportive care, complete remission (CR) rates as high as 50% to 80% can be achieved (Mayer et al., 1994, Paietta, 2012) in adult patients with AML. However, despite this success half of the patients will eventually relapse due to the persistence of residual malignant cells surviving after chemotherapy. The persistence of residual malignant cells below the threshold of conventional morphological findings is termed minimal residual disease (MRD) and may identify patients at a higher risk of relapse (Venditti et al., 2000, Buccisano et al., 2009, Lane et al., 2008).

In this setting, the aim of monitoring MRD is very important for:

- monitoring the effectiveness of treatment in order to give individual information on disease prognosis and to design patient adapted post-remission therapies. Especially for the group of “standard risk” patients, who are experiencing great heterogeneity in treatment response,
- identification of cases with a high risk of relapse that then can be treated earlier and more effectively,
- determining patients who will benefit from bone marrow transplantation (BMT),
- assessing the effectiveness of new treatments.

Hence, detection of low levels of malignant cells with molecular techniques has become a key tool of contemporary haematological diagnostics. The final goal of detecting MRD is to obtain an early evaluation of the effectiveness of the treatment and possibly provide pre-emptive therapy, as it is currently applicable for APL (Grimwade and Tallman, 2011, Paietta, 2012, Hourigan and Karp, 2013).

1.5.2 Techniques for MRD assessment

Since MRD means the presence of leukemic cells among normal cells, techniques used for MRD detection rely on finding leukemia-specific markers, which distinguish the leukemic blasts from the normal cells. Currently, specific translocation markers are available for approximately 25% of AML patients and these include fusion genes, like *RUNX1-RUNX1T1* and *PML-RARA* (Bhatia et al., 2012). With the detection of gene mutations, such as *NPM1* (Papadaki et al., 2009) this spectrum will widen.

For this purpose various techniques have been developed, which differ in specificity of the markers used, as well as in the detection levels. Each method has relative advantages and disadvantages (Radich and Sievers, 2000), but some of them, like morphology of the cells and conventional cytogenetics, are limited by their low sensitivity. Cytomorphology is still a standard technique for identification of complete remission but the detection limit is 10^{-1}-10^{-2}. It is based on the assessment of morphology of bone marrow cells with the use of a light microscope (Toren et al., 1996). Sensitive methods to detect MRD include the "classic" metaphase cytogenetics, cell cytometry studies and molecular genetic studies such as polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH). However, techniques other than PCR are inferior due to low sensitivity. The higher sensitivity of PCR enables detection of 1 leukemic cell among 10^{-5}-10^{-6} normal cells (Willemse et al., 2002). PCR-based techniques allow the detection of leukemia-specific gene rearrangements by identifying either leukemia specific translocations or clone-specific immunoglobulin heavy chain (*IGH*) gene and T-cell receptor (*TCR*) gene rearrangements. Therefore, nowadays detection of MRD by PCR has become an essential tool for molecular monitoring of AML (Geng et al., 2012, Jourdan et al., 2013, Paietta, 2012) and it can be quantified by the use of Reverse-Transcriptase PCR (RT-PCR) or the nested-PCR and quantitative PCR: Real time Quantitative PCR (RQ-PCR). MRD quantification can be carried out either by the end point (competitive) RT-PCR or the cycle-cycle (real-time) techniques.

RQ-PCR can be used for MRD detection in the following cases:

- detection of fusion genes like *RUNX1-RUNX1T1*, *CBFB-MYH11* and *PML-RARA*
- detection of mutations of specific genes like *NPM1*,
- detection of genes which are pathologically expressed like Wilms tumor (*WT1*) gene and Ecotropic viral integration-1 (*EVI1*) gene.

2. AIM OF THE STUDY

AML is a disease with wide clinical and biological diversity. During the last decade much progress has been made in understanding the molecular and cytogenetic basis of acute leukemia. This complexity of the genetic findings has been taken into account in the last published WHO classification of AML (Vardiman et al., 2009).

AML with t(8;21) belongs to the CBF leukemias and is associated with a favourable prognosis. However, despite the improved rates of CR, between 25% and 30% of patients will relapse with current treatment protocols (Yin et al., 2012, Leroy et al., 2005). Therefore, identifying patients at a higher risk of relapse and thus preventing it, is of major clinical importance. Several studies (Leroy et al., 2005, Paietta, 2012, Zhu et al., 2013, Yin et al., 2012, Schnittger et al., 2003) have suggested that the molecular detection of residual leukemic blasts below the threshold of conventional morphological findings for CR (<5% blasts in the bone marrow), might be the most suitable method for the successful identification and prevention of relapse.

The aim of this study is to establish a real time PCR assay for sensitive and specific *RUNX1-RUNX1T1* quantification in relation to the Abelson gene (*ABL1*) as a reference housekeeping gene, which is suitable for monitoring MRD in routine diagnostics. Furthermore based on the finding that coexistence of *JAK2*V617F and *RUNX1-RUNX1T1* mutation has a frequency of approximately 4%-6% in *de novo* AML (Steensma et al., 2005, Dohner et al., 2006), the presence of additional *JAK2*V617F mutation was examined in the sample material of the study using DNA melting curve analysis.

3. MATERIALS AND METHODS

3.1 Material

3.1.1 Oligonucleotides

The oligonucleotides were purchased from Metabion (Munich).

***RUNX1-RUNX1T1* RQ-PCR**

RUNX1-RUNX1T1 primers and probes:
Forward primer: 5'-CACCTACCACAGAGCCATCAAA-3'
Reverse primer: 5'-ATCCACAGGTGAGTCTGGCATT-3'
TaqMan probe: 5'-6-FAM-AACCTCGAAATCGTACTGAGAAGCACTCCA-BHQ1-3'

ABL1 primers and probes:
Forward primer: 5'-CCTTTTCGTTGCACTGTATGATTT-3'
Reverse primer: 5'-GCCTAAGACCCGGAGCTTTT-3'
TaqMan probe: 5'-6-FAM-TGGCCAGTGGAGATAACACTCTAAGCATAACTAA AGG-BHQ1-3'

***RUNX1-RUNX1T1* primary and nested PCR**

RUNX1-RUNX1T1 primers

Primary PCR
AMLex: 5’-GAGGGAAAAGCTTCACTCTG-3’
ETOex: 5’-TCGGGTGAAATGTCATTGCG-3’
Nested PCR
AMLint: 5’-GCCACCTACCACAGAGCCATCAAA-3’
ETOint: 5’-GTGCCATTAGTTAACGTTGTCGGT-3’

ABL1 primers
Forward primer: 5’-GGCCAGTAGCATCTGACTTTG
Reverse primer: 3’-ATGGTACCAGGAGTGTTTCTCC

Melting curve analysis for *JAK2*V617F
Forward primer: 5'- AAGCAGCAAGTATGATGAG-3'
Reverse primer: 5'- CCCATGCCAACTGTTTAG-3'

Hybridization Probes:
JAK2-A: 5'-AGTGATCCAAATTTTACAAACTCCTGAACCAGAA-FL-3'
JAK2-S: 5'-LC-Red-640-TTCTCGTCTCCACAGACACAT-P-3'

3.1.2 Mammalian cell lines

In this work we used Kasumi-1 cell line (Human acute myeloid leukemia, Source: ACC220, DSMZ, Braunschweig, Germany), in order to establish a PCR method for the quantification of the *RUNX1-RUNX1T1* transcripts. The cell line was used for the establishment of standard curves and as a positive control.

Kasumi-1 is a cell line that was isolated in 1989 from the PB of a 7 years-old Japanese boy with AML (AML FAB M2). These cells carry the t(8;21) translocation that leads to the formation of *RUNX1-RUNX1T1* fusion gene.

The following cell lines were used as negative controls:

1) K562, established from a patient with chronic myelogenous leukemia (CML) in blast crisis,

2) ME1, derived from patient with AML (AML FAB M4Eo),

3) NB-4, derived from patient with APL,

4) OCI, derived from patient with acute myelomonocytic leukemia and

5) SD1, derived from the PB of a patient with *BCR-ABL* positive acute lymphoblastic leukemia (ALL).

3.1.3 Patients

Patient samples were referred to the Laboratory for Leukemia Diagnostics, Department of Medicine III, Klinikum Großhadern, Munich, for routine cytogenetic and molecular analysis. Based on available sample material, we used BM samples from 37 AML patients and PB samples from 2 AML patients, from the cohort of AMLCG99 study population which were diagnosed positive for the *RUNX1-RUNX1T1* fusion gene encoded by translocation t(8;21)(q22;q22). The diagnosis was established in the Laboratory for Leukemia Diagnostics using molecular and cytogenetic analysis. 29 *RUNX1-RUNX1T1* positive samples were available at diagnosis, 12 samples at day 16 of the induction therapy, 143 at various points during follow up and finally 5 *RUNX1-RUNX1T1* positive samples at relapse. Diagnosis of AML was made morphologically and cytochemically as it has been previously described (Kern et al., 2003) and was based on FAB classification. All patients had been treated according to the therapeutic AMLCG99 protocol (Buchner et al., 1999, Buchner et al., 2003, Buchner et al., 2006). All patients had given their informed consent before entering the study. Table 4 and Table 5 provide the clinical and cytogenetic data of the patients included in the study at the time of diagnosis. As described in Table 6, in addition to the mutations that had already been studied, we also screened our *RUNX1-RUNX1T1* positive patients for the detection of *JAK2*V617F mutation using melting curve analysis.

Table 4: Patients' characteristics (n=39).

Age (years)	
Range	15.8-74.8
Median	45.92
WBCsx10^3/µl	
Range	0.98-50.610
Median	10.500
PLTx10^3/µl	
Range	4-273
Median	29
Blasts at diagnosis (n=37)	
Range	25-95%
Median	75%
FAB subtype	
M1	2
M2	37

Abbreviations: FAB, French-American-British classification; PLT, platelet count; WBCs, white blood cells

Table 5: Cytogenetics at diagnosis.

Cytogenetics	Number
Sole t(8;21)	11
Loss of X or Y	19
Del(9)(q22)	3
Additional aberrations	6

Table 6: Additional mutations

***FLT3*-ITD**		***MLL*-PTD**	
+	2	+	0
-	36	-	39
Not done	1		
***FLT3*-D835**		***KIT*- D816**	
+	1	+	5
-	36	-	31
Not done	2	Not done	3
		***JAK2*V617F**	
		+	0
		-	18
		Not done	21

3.1.4 Chemicals and Kits

Cell culture	
RPMI 1640 medium	(PANBiotech, Aidenbach)
Fetal calf serum 10% (FCS)	(Biochrom AG, seromed, Berlin)
Penicillin/Streptomycin	(GIBCO, Germany)
PBMCs cell separation	
Phosphate Buffer Saline (PBS)	(Dulbecco Biochrom AG, Berlin)
Biocoll separating solution	(Biochrom AG, Berlin)
Quicklyser-II	(Sysmex, Norderstedt)
RNA isolation	
RLT buffer	(Qiagen, Hilden)
QIAshredder	(Qiagen, Hilden)
MagNA Pure LC mRNA Isolation KIT	(Roche, Mannheim)
cDNA Synthesis	
Desoxynucleotide (dNTP's)	(Invitrogen, Karlsruhe)
dNTPs-Mix (10 mM)	(Promega, Mannheim)
Random hexamers primers $p(dN)_6$	(Roche Diagnostics Mannheim)
RNase Inhibitor	(Promega, Mannheim)
Superscript II (Reverse Transcriptase)	(Invitrogen, Karlsruhe)
Gel electrophoresis	
Agarose	(UltraPure, Invitrogen)
DNA molecular weight marker VI	(Roche Diagnostics, Mannheim)
Ethidium bromide 1% (10 mg/ml)	(Carl Roth, Karlsruhe)
Loading dye 6x	(Promega, Mannheim)
10x TBE buffer	(Roche, Mannheim)
PCR	
Taq polyme*RASe*	(Qiagen, Hilden)
dNTPs	(Invitrogen, Karlsruhe)
LightCycler TaqMan Master Mix	(Roche Diagnostics, Mannheim)
LightCycler Fast Start DNA Master HybProbe	(Roche Diagnostics, Mannheim)
Kits	
MagNA Pure LC mRNA Isolation KIT	(Roche, Mannheim)
LightCycler TaqMan Master Mix	(Roche Diagnostics, Mannheim)
LightCycler Fast Start DNA Master HybProbe	(Roche Diagnostics, Mannheim)

3.1.5 Laboratory equipment

Cell culture incubator	(WTB, Tuttlingen)
Centrifuger Rotanta 460R	(Hettich, Germany)
Cell culture CO_2 incubator	(Heraus, Osterode)
Eppendorf centrifuge 5415D	(Eppendorf,Hamburg)
Eppendorf cups (0.5-1.5 ml)	(Eppendorf, Hamburg)
Eppendorf® tabltop centrifuge 5415D	(Eppendorf, Hamburg)
Electrophoresis champer	(Horizon 11-14, GIBCO BRL, USA)
Falcon tubes®	(Becton Dickinson, Biosciences)
Fridge (4°C, -20°C)	(Siemens AG, Erlangen)
Fridge (-80°C) UF80-450S	(Colora Messtechnik GmBH, Lorch)
Gel electrophoresis systems	(Bio-rad, Munich)
LightCycler™ real-time PCR machine	(Roche Diagnostics, Mannheim)
MagNA Pure LC	(Roche Diagnostics Mannheim)
Microcell counter	(Sysmex, Norderstedt)
Pipette Accu-jet	(Brand, Wertheim)
Pipette tips	(Star Labs, Munich)
Pipettes	(Gilson, Langenfeld and Eppendorf, Hamburg)
Pipettes, Tissue culture flasks, Centrifuge vials	(Sarstedt, Nümbrecht)
Thermocycler Cyclone 25	(Peqlab Biotechologie, Erlagen)
Thermocycler T3	(Biometra)
Vortex	(Scientific industries Bohemia USA)

3.1.6 Software

Adobe Illustrator	(Adobe Systems, Unterschleißheim)
Adobe Photoshop	(Adobe Systems, Unterschleißheim)
EndNote 6.0.2	(Thompson ISI, Carlsbad, CA, USA)
Microsoft Office 2003	(Microsoft, Redmond, WA, USA)
SigmaPlot 6.0	(SPSS Incorporated, Chicago, USA)
LightCycler SW Version 3.5 and 4.05	(Roche Diagnostics, Mannheim)

3.2 Methods

3.2.1 Cell culture

Cells were cultured in RPMI-1640 medium with 10% heat inactivated Fetal Bovine Serum (FBS) supplemented with 5 U/ml of penicillin and streptomycin at 37°C under a humid condition in 5% CO_2.
They were suspended in the medium to reach a final cell concentration of $1x10^6$ cells/ml. Every 2 or 3 days saturated cultures were divided at a ratio of 1:2 to 1:3.

3.2.2 Isolation of PB mononuclear cells

The isolation of peripheral blood mononuclear cells (PBMCs) was performed with gradient density centrifugation, using Biocoll separating solution. Ficoll has a higher density than lymphocytes or monocytes and a lower density than erythrocytes and granulocytes. By centrifugation, monocytes, lymphocytes and natural killer cells (PBMCs) are enriched in the interphase layer between whole blood/bone-marrow and the Ficoll solution and can be recovered by pipetting.
15 ml of Biocoll separating solution (density = 1.077 at +20°C) was placed in 50 ml centrifuge tubes. 5-10 ml of heparinized BM or whole blood were mixed with an equal volume of phosphate buffer saline (PBS) in 50 ml centrifuge tubes and then were applied over Biocoll separating solution using a sterile 10 ml pipette, with caution. Centrifugation at 1200 g (without brake) for 20 min at room temperature was followed. The layer of mononuclear cells, formed between the aqueous face and the Ficoll was collected using a 10 ml disposable pipette. The cells were then carefully transferred to a 50 ml vessel and washed with 1xPBS. The supernatant after a 10 min centrifugation at 300 g was discarded. Cell counting was performed using the Microcell counter. Aliquots of $10x10^6$ cells (samples during diagnosis and follow up) were then prepared and immediately lysed in 300 µl of RLT buffer. The RLT lysates were stored in 1.5 ml centrifuge tubes at -80°C.

3.2.3 RNA extraction

3.2.3.1 RNA extraction from patient samples

RNA isolation from PBMCs was manually performed using the MagNA Pure LC mRNA isolation KIT, according to the manufacturer protocol with minor modifications. In brief, the RLT lysates were initially thawed at room temperature. The cells were washed twice with ice cold PBS, RLT lysis buffer (250 µL) was added to the cell

pellet and homogenization was performed using QIAshredder. RNA was extracted using MagNa Pure Nucleic Acid Purification System, according to the manufacturer's instructions. Final elution of mRNA was performed in a volume of 30 µl.
RNAse free disposables (test tubes and pipette tips) were used during processing RNA.

3.2.3.2 RNA extraction from Kasumi-1 cell line

$3x10^6$ Kasumi-1 cells were lysed in 300 µl RLT buffer. mRNA extraction was carried out using the same protocol as described for the patients samples.

3.2.4 cDNA synthesis

Isolated mRNA was reversely transcribed to complementary DNA (cDNA) using Superscript II reverse transcriptase (Invitrogen Karlsruhe, Germany).
10 µL of mRNA extracted from Kasumi-1 cell line and/or from samples at diagnosis were used in the reverse transcription (RT) reaction.
For MRD detection cDNA synthesis was performed using 30 µL of mRNA, extracted from approximately $10x10^6$ cells.
RNA samples were initially denatured in 70°C for 8 min and then cooled down to 4°C prior to adding the RT Mastermix in a final volume of 50 µl.
RT MasterMix was prepared as follows:

Table7: RT-MasterMix

MasterMix cDNA synthesis	Volume
5x First-Strand Buffer	10.0 µl
dNTPs (10 pmol/µl)	4.4 µl
Random Primer (50 µg/µl)	2.5 µl
RNasin (40 U/µl)	1.25 µl
SuperScript II RT (200 U/µl)	1.9 µl
RNase-free water	*up to 20 or 40 µl*

MasterMix was then added to each RNA sample and RT was performed at 37°C for 60 min. The reaction was stopped by heat inactivation of the enzyme at 95 °C for 5 min.
ABL1 gene amplification was performed for each sample in order to control the RNA integrity (Schoch et al., 2002). Strict precautions were taken in order to prevent cross contamination. As negative control RNA derived from *RUNX1-RUNX1T1* negative cell lines was used (paragraph 3.1.2) RNAse-free water was also used as a non-template control. Finally, amplification products were analysed on 2% agarose gels stained with ethidium bromide.

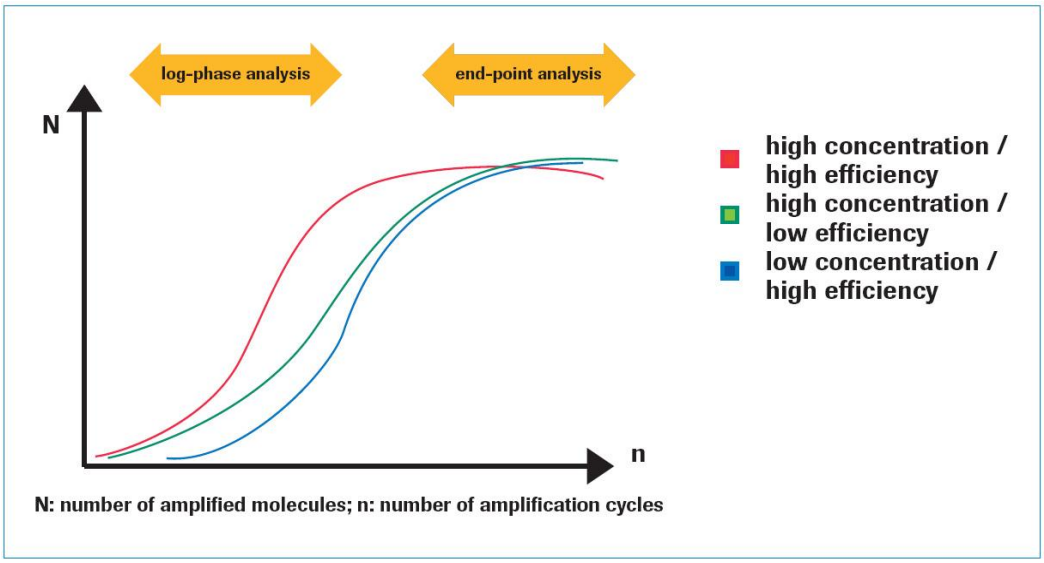
3.3 Principle of PCR

PCR is the most sensitive and widely used technique in MRD detection. It was first described in the mid-1980s by Kary B. Mullis (Mullis and Faloona, 1987). The technique is based on the enzymatic amplification of a DNA fragment that is bounded by two primers. Primers are oligonucleotides that are complementary to the target sequence and bind specifically to it. The DNA portion bounded by the two primers is used as a template for the construction of the complementary strand.

The reaction requires deoxynucleotide triphosphates (dNTPs) which are used to create the cDNA strand and is catalysed by a thermostable DNA polymerase, (Taq polymerase, of the species Thermus aquaticus). For RNA quantification, RT takes place as a first step before PCR, in order to convert RNA into cDNA. The cDNA can be stored for a long time. It is commonly accepted that RNA is extremely unstable (compared to DNA). Thus collection, storage and transport of the samples have to take place with great caution to avoid contamination and to ensure the integrity of the samples (Valasek and Repa, 2005).

PCR is a chain reaction of repeated cycles, with each cycle consisting of three steps: denaturation of double strand DNA at about 95^0C, primer annealing at about 63^0C (depending on the primer sequence) and extension/elongation step, where synthesis of the new strand occurs at 72^0C. After 20 cycles, roughly 1 million copies of the target DNA sequence are produced. After a number of cycles the exponential phase reaches the plateau phase due to accumulation of end-product inhibitors or depletion of the substrates. The detection of the PCR products at the plateau phase of the PCR reaction (end-point detection) cannot lead to a correlation between the amount of PCR product and the DNA quantity used as a template in the PCR reaction. The first attempts for quantifying the DNA template were based on end-point analysis (competitive PCR).

Figure 5 : Principle of PCR



(adapted from Roche Applied Science Technical Note No. LC 10/update 2003)

3.3.1 Principle of real-time PCR

Real-time PCR offers an alternative method for both qualitative and quantitative analysis. The principle of this technique is to estimate the levels of PCR products as these accumulate at the exponential phase of the amplification, rather than estimating the level of the final products (competitive RT-PCR). The detection of the product depends on the fluorescent signals which are produced during the reaction. In the quantitative RT-PCR the fluorescent signal measured at each amplification cycle is correlated to the amount of PCR product formed, and finally is converted into a numerical value for each sample.

In this study, RQ-PCR was performed using LightCycler instrument 1.5. In this apparatus, PCR occurs in special glass capillaries which are placed into a carousel, and air is used for fast heating and cooling (Wittwer et al., 1997). A micro-volume fluorimeter is used to quantify the amplification products and the whole reaction is recorded in the screen of the connected PC and analysed using the appropriate software.

The fluorescent signal increases exponentially during the amplification phase of the PCR reaction. In the amplification reaction, the cycle at which the fluorescence of the sample rises above the background is called the Crossing Point (CP) (which is usually determined at the first 3-15 cycles of the reaction). Quantification in real-time PCR involves the determination of the CP of a sample.

3.3.1.1 Detection of the PCR products

The fluorescent signal can be produced using different assay methods:

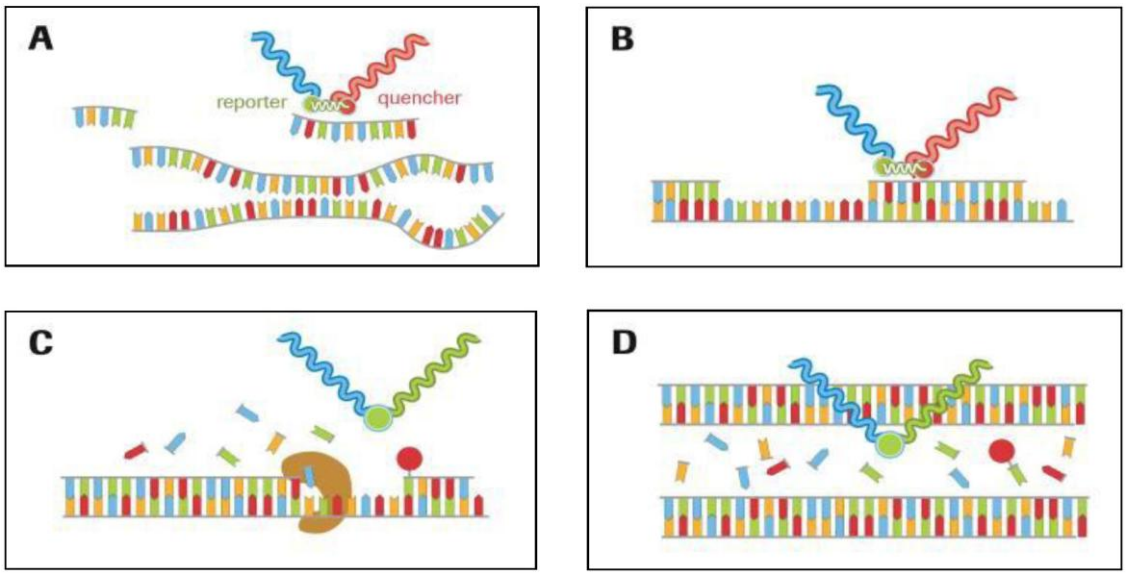
i) Sequence-independent detection assays (typically SYBR Green I) and

ii) Sequence-specific probe binding assays (hydrolysis probes, hybridization probes).

The LightCycler offers several formats for detection of the PCR products, including hydrolysis or TaqMan probes, which were applied in this work. This probe is an oligonucleotide with a reporter dye attached at the 5' end and a quencher dye at the 3' end. As long as the probe is intact, the fluorescent dyes are close to each other and the signal produced from the reporter dye is "suppressed" by the quencher dye. The fluorescent quenching is due to Fluorescence Resonance Energy Transfer (FRET) (Clegg, 1995).

The hydrolysis probes emit fluorescence when 5'-3' exonuclease activity of Taq polymerase degrades the TaqMan probe. In this way reporter and quencher are separated and fluorescent dye is released. The amount of the PCR product is directly proportional to the increase of the fluorescence of the reporter dye measured. Figure 6 shows schematically the principle of hydrolysis probes. As mentioned above TaqMan probes are cleaved during the PCR assay, so they cannot be used to perform melting curve analysis (Wittwer et al., 1997, Bustin, 2000).

Figure 6: Principle of hydrolysis



(adapted from Roche Applied Science Technical Note No. LC 18/2004)

3.3.2 Relative quantification using LightCycler technology

Relative quantification is defined as the ratio of target DNA to a reference gene (housekeeping gene). The reference gene is a gene that is expressed constitutively at the same level in all samples analysed.

$$\text{Relative ratio} = \frac{\text{target gene concentration}}{\text{Reference gene concentration}}$$

3.3.2.1 Relative quantification calibrator normalized

In this method, the absolute value of CP is used in order to calculate the normalized value of the amount of the target gene in relation to a calibrator. At first, the CP difference (ΔCP) between the housekeeping and the target gene is calculated for both, the sample and the calibrator ($\Delta\Delta CP = \Delta CP_{sample} - \Delta CP_{calibrator}$). Finally based on the equation $2^{-\Delta\Delta CP}$ the normalized value of the target gene in relation to the calibrator is calculated.

3.3.2.2 Relative quantification with external standards

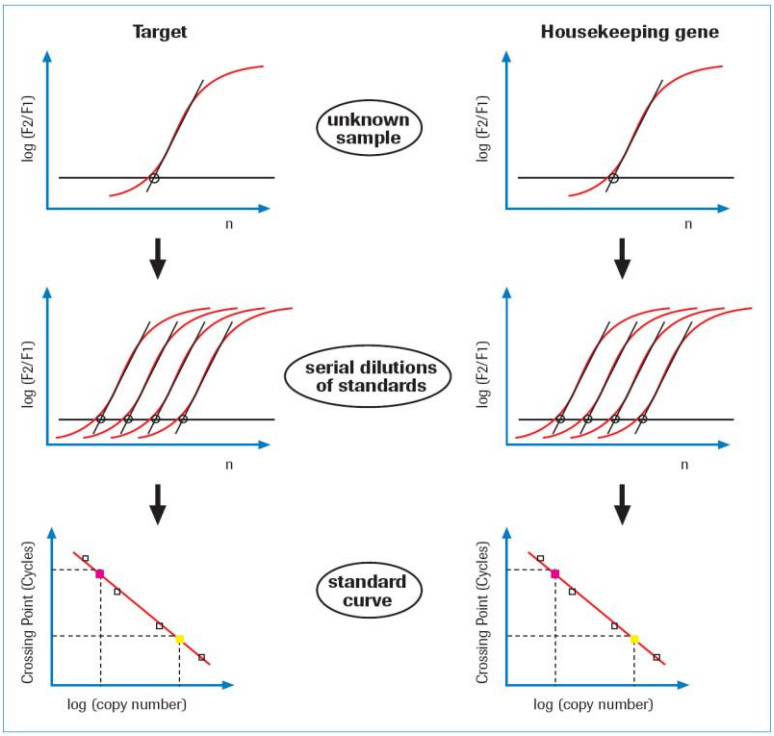
Serial dilutions of DNA standards for reference and target genes are used to create standard curves. Sample and calibrator CP values are analysed using the corresponding standard curve in order to be quantified.

Then the target gene is normalized to the reference gene, by dividing the amount of the target gene to the amount of the housekeeping gene.

$$\text{Calibrator - normalized ratio} = \frac{\text{Ratio target / reference (sample)}}{\text{Ratio target / reference (calibrator)}}$$

The two genes cannot be amplified with the same efficiency, since PCR efficiency is influenced by target-specific factors, such as primer annealing, GC-content and product size. The optimum PCR efficiency is 2 (E=2) which means that the amount of PCR product duplicates during each cycle. This corresponds to a slope of -3.32 of the standard curve. The slope of that curve can be directly converted into efficiency using the formula: $\mathbf{E = 10^{-1/slope}}$.

Figure 7: Principle of relative quantification with external standards



(adapted from Roche Applied Science Technical Note No L.C 10/update 2003)

In order to create standard curves for the reference and the target gene, serial dilutions of cDNA from Kasumi-1 cell line was used. All samples were assayed in duplicates. In all experiments Kasumi-1 cell line was used as a positive control, *RUNX1-RUNX1T1* negative cell line as negative control and RNAse-free water as a non template control. Results were analysed using the LightCycler SW4.5. The RQ-PCR reaction was carried out in a total volume of 20 µl per capillary. The MasterMix was prepared as follows:

Table 8 : RQ-PCR reaction mix

MasterMix PCR	Concentration	Volume/capillary
RNAse free H2O		11.6 µl
Probe 10 µM	0.2 µM	0.4 µl
Forward primer 10 µM	0.5 µM	1 µl
Reverse primer 10 µM	0.5 µM	1 µl
LC TaqMan Master Mix	1x	4 µl
Volume		**18 µl**
cDNA		**2 µl**

RQ-PCR for *RUNX1-RUNX1T1* was performed in the LightCycler using the following conditions:

Table 9: RQ-PCR protocol

Analysis mode	Cycles	Segment	Target Temperature	Hold Time	Acquisition Mode
Pre-Incubation					
None	1		95°C	10 min	none
Amplification					
Quantification	50	Denaturation	95 °C	10 s	none
		Annealing	63 °C	30 s	none
		Extension	72 °C	01 s	single
Cooling					
None	1		40 °C	30 s	none

The analysis was displayed in the fluorescent channels F1/F3 (530/705 nm).

3.3.3 Qualitative primary/nested PCR for the detection of the *RUNX1-RUNX1T1* hybrid gene

To perform primary and nested PCR reaction (qualitative PCR), 1, 5, and 10 µl of cDNA, that was transcribed as described in paragraph 3.2.4, was used.

The primary PCR reaction was performed in a final volume of 50 µl containing 0.5 µM of each primer under the cycling conditions described in Table 10. Primers' sequences were described previously (Miyamoto et al., 1997) and are shown on page 23. 5 and 10µl of the first PCR product were used as a template for the nested PCR reaction using nested primers as listed on page 23. Cycling conditions were the same as for the primary PCR reaction. Each PCR reaction contained a positive control from Kasumi-1 cell line and RNAse-free water as a non template control.

The sensitivity of the primary and the nested PCR was assessed using ten-fold cDNA dilutions from the Kasumi-1 cell line. Established guidelines to prevent PCR contamination were stringently followed.

ABL1 amplification was used to check RNA integrity in all patient samples. Following the guidelines given by the Europe Against Cancer (EAC) program (Beillard et al., 2003), *ABL1* was used as a reference gene, since it is constantly expressed in all investigated samples. Importantly *ABL1* gene doesn't contain any pseudogene.

All patient samples were tested in duplicate.

Primers are listed in paragraph 3.1.1 (page 23).

Table10:. Conditions of the primary and nested PCR for *RUNX1-RUNX1T1* and *ABL1* .

PCR program		
94°C	5 min	
94°C	60 sec	
62°C	60 sec	35 cycles
72°C	60 sec	
72°C	10 min	

3.4 Melting curve analysis

3.4.1 Principle of melting curve analysis

The melting temperature (Tm), is the temperature at which 50% of the DNA becomes single stranded. Tm is specific for each double-stranded DNA (ds DNA) because it is primarily dependant: a) on the length of the dsDNA, b) the degree of the GC content (Tm is higher in GC-rich fragments) and c) on the degree of complementarity between the strands. This is why melting curve analysis is able to distinguish PCR products with the same length but different GC/AT ratio. Therefore, the method can be applied for mutation analysis, such as point mutations or small deletions.

In melting curve analysis, hybridization probes can be used. Hybridization probes are two specifically designed, sequence-specific oligonucleotide probes, labelled with different dyes. After hybridization, in the annealing phase, these probes are designed to bind to the amplified DNA fragment in a head-to-tail orientation, bringing the two dyes into close proximity. Consequently, the emitted energy excites the acceptor dye attached to the second hybridization probe, which then emits fluorescent light at a different wavelength.

In mutation analysis, a pair of hybridization probes, complementary to the wild-type sequence, is used. In cases where mutant sequence is amplified the probe binds to the DNA with a mismatch, which results in a 5°C decrease of the Tm.

After PCR amplification, the hybridized products are slowly heated with continuous measurement of the fluorescent signal until the point that the probes are not in close proximity anymore and the fluorescent signal decreases. The “mutation” probe dissociates at a different temperature and the melting point is shifted. That means that every mutation has its own melting curve. If there is only one mutant allele, the melting curve shows two peaks, one corresponding to the mutant allele and the other to the wild type.

3.4.2 Melting curve analysis for the detection of the *JAK2* gene mutation (V617F)

Screening of *RUNX1-RUNX1T1* positive patients for the presence of *JAK2*V617F mutation was performed using melting curve analysis, on the LightCycler instrument 1.5 (Roche Diagnostics, Mannheim, Germany) and the results were analyzed with LightCycler SW 4.5. Sequences of primers and probes are shown in paragraph 3.1.1 (page 23). The PCR reaction MasterMix is listed in Table 11 and the cycling conditions for the melting curve analysis are presented in Table 12.

Table 11: MasterMix for Melting curve analysis

MasterMix	Concentration	Volume/capillary
RNAse free H2O		9.6 µl
$MgCl_2$	4 mM	2.4 µl
Hyb-probe S	0.75 µM	1 µl
Hyb-probe A	0.75 µM	1 µl
Left primer	0.5 µM	1 µl
Right primer	0.5 µM	1 µl
LightCycler-FastStart DNA Master	1x	2 µl
MasterMix		**18 µl**
cDNA		**2 µl**

Table 12: Cycling conditions for Melting Curve analysis

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time	Acquisition Mode
Pre-Incubation					
None	1		95°C	10 min	none
Amplification					
Quantification	40	Denaturation	95°C	1 sec	none
		Annealing	60°C	10 sec	single
		Extension	72°C	10 sec	none
Melting Curve					
Melting curve	1		95°C	1 min	none
			40°C	20 sec	none
			85°C	0 sec	continuous
Cooling					
None	1		40°C	1 min	none

The analysis was displayed in the fluorescent channels F2/F1 (640/530 nm) (Schnittger et al., 2006a).

3.5 Agarose gel electrophoresis

2g of agarose powder were added to 100 ml of electrophoresis buffer (1xTBE) (2%) and then the mix was heated in a microwave oven until agarose dissolved. After the cooling of the solution to 50^{o}C, 3.5 µl ethidium bromide (0.35 µg/ml) was added and the warm solution was poured into a tray and let to cool in room temperature for 30-40 minutes. Then the gel was placed in an electrophoresis chamber and covered with 1xTBE buffer. 5 µl of loading dye was added to 20 µl of PCR product and placed into the well. A DNA molecular weight marker (0.15-2.1 kbp) was used as a ladder for size reference and the agarose gel electrophoresis was run at 140 Volts for approximately 40 min, then the gel was visualized and photographed under UV light. Primary and nested PCR products were separated and visualized after agarose gel electrophoresis.

4. RESULTS

4.1 Positive control

As a *RUNX1-RUNX1T1* positive control, the Kasumi-1 cell line was used. 10µL of mRNA extracted from approximately 10^6 cells of Kasumi-1 cell line were used for cDNA synthesis, as already described in the method section (3.2.4, page 29). Kasumi-1 cDNA concentration was pooled, aliquoted and frozen at a concentration of 1771.0 ng/µl. For the establishment of the LightCycler-PCR assay and the preparation of the standard curves (target, reference gene), 10-fold serial dilutions of cDNA Kasumi-1 cell line were used. The dilutions were prepared in TE buffer (10 mM Tris, 1 mM EDTA, pH:7) as follows: 1:10, 1:100, 1:1000, 1:10000, 1:50000, 1:100000 and 1:1000000. In PCR, 10 µl of the 10fold dilution of the Kasumi-1 cDNA in TE were used.

4.2 Establishment of the Real-time Quantitative PCR for *RUNX1-RUNX1T1* quantification

4.2.1 Relative quantification – Reference gene

The principle of relative quantification has been described in the section 3.3.2 (page 33). To create the assay, and to compensate variations of RNA amount and integrity, *RUNX1-RUNX1T1* fusion transcript was normalized to a reference gene, the housekeeping gene *ABL1*. *ABL1* was chosen according to “Europe Against Cancer (EAC) Program” (Gabert et al., 2003).

$$\text{Relative ratio} = \frac{\textit{RUNX1-RUNX1T1}\ \text{concentration}}{\textit{ABL1}\ \text{concentration}}$$

4.2.2 Calibrator

To normalize sample values within a run and between runs, the *RUNX1-RUNX1T1/ABL1* expression ratios in all samples were divided by the *RUNX1-RUNX1T1/ABL1* expression ratio of a calibrator. cDNA of the Kasumi-1 cell line was used as a calibrator:

$$\text{Calibrator normalized ratio} = \frac{\textit{Ratio}\ \ \textit{RUNX1-RUNX1T1}\ /\ \textit{ABL1}\ \text{(sample)}}{\textit{Ratio}\ \ \textit{RUNX1-RUNX1T1}\ /\ \textit{ABL1}\ \text{(calibrator)}}$$

4.2.3 Primers and Probes

The oligonucleotides for RQ-PCR were designed using the Primer Express 2.0 software (PE Applied Biosystems).

In order to design primers and probes special guidelines were taken into consideration (Bustin, 2000). Primers and probes should have G/C content between 30% to 80% and a balanced distribution of G/C and A/T bases rich domains. Probes should have a Tm that allows annealing at 5-10^{o}C higher than the Tm of the primers. Length of Primers should be between 20-24 bp and length of TaqMan probes 30 bp according to recommendations in the Roche Applied Technical Note No. LC 11/update 2003.

The primers' and probes' sequences for the quantification of *RUNX1-RUNX1T1* are in accordance to the "Europe Against Cancer Program" (Gabert et al., 2003) (GenBank Accession Numbers D43969 (*AML1*) and D14289 (*ETO*). Sequences are listed in paragraph 3.1.1 (page 23) and have the following characteristics:

Forward primer: GC% = 50% and Tm = 60.3^{o}C

Reverse primer: GC% = 50% and Tm = 60.3^{o}C

TaqMan probe: GC% = 47% and Tm = 66.8^{o}C

ABL1 was the recommended housekeeping gene, according to the "Europe Against Cancer Program" (Gabert et al., 2003).

GenBank Accession Number M14752 was used in order to design the primers and probe with the software mentioned above.

Primers and probes for *ABL1* have the following characteristics:

Forward primer: GC% = 38% and Tm = 58^{o}C

Reverse primer: GC% = 55% and Tm = 58^{o}C

TaqMan probe: GC% = 43% and Tm = 70^{o}C

4.2.4 Optimization of RQ-PCR protocol for *RUNX1-RUNX1T1*

In order to set up and optimize the runs on the LightCycler, the LC Software Short Guide Version 3.3, April 2000 as well as the LC Operator´s Manual Version 3.5, October 2000 were used. PCR conditions were optimized using cDNA from the Kasumi-1 cell line in order to achieve the highest possible efficiency, specificity and sensitivity.

For example, annealing temperatures at 60, 61, 62, 63, 64, and 65^{o}C and an annealing time range between 15 to 40 sec, were tested. The highest efficiency and specificity was achieved at 63^{o}C and 30 sec, as it was shown by gel electrophoresis. The results were not specific when using lower annealing temperature or a shorter annealing time. The initial denaturation was performed at 95 ^{o}C for 10 min, followed by 50 amplification cycles of denaturation at 95 ^{o}C for 10 sec, annealing of the primers for 30 s at 63^{o}C and extension at 72 ^{o}C for 1 sec. Finally, the reaction was cooled down to 40 ^{o}C for 30 sec. The final protocol of RQ-PCR, is also presented in Table 9, page 35.

4.2.5 PCR protocol for amplification of the reference gene *ABL1*

PCR reactions for the reference gene (*ABL1*) were carried out under the same conditions as described for the target gene (*RUNX1-RUNX1T1*). This represents an important advantage for routine diagnostics meaning that target and reference gene can be included in one run.

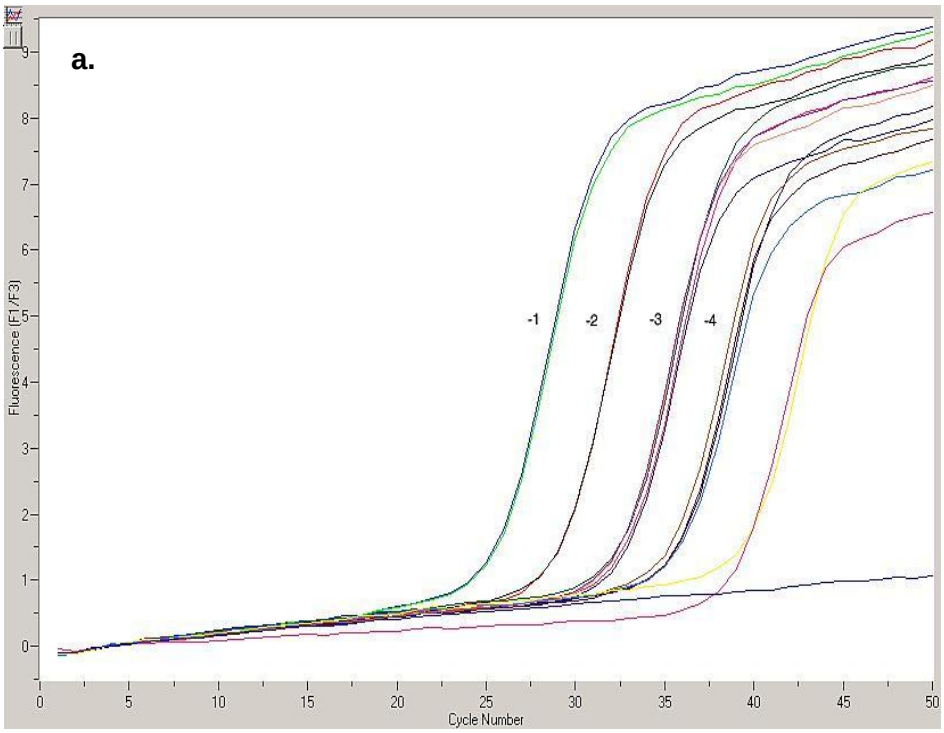
4.2.6 Creation of the standard curves

Standard curves for the target and reference gene were prepared separately by performing 10 fold dilutions (10^{-6} to 10^{-1}) of cDNA from Kasumi-1 cells.

To create a standard curve for *RUNX1-RUNX1T1*, the cDNA from Kasumi-1 cells were diluted as follows: 1:10, 1:100, 1:1000, 1:10000, 1:50000 and each of these dilutions were analyzed 2 to 6 times.

To create the standard curve for the reference gene the following concentrations were used: 1:10, 1:100, 1:1000, and 1:10000. Each dilution was analyzed 6 times. Finally, a good linearity and reproducibility of the standard curve was obtained for both the *RUNX1-RUNX1T1* and *ABL1* assay (Fig 8).

Figure 8: Amplification plots of 10-fold dilutions of Kasumi-1 cell line cDNA: a) Target gene amplification curves b) reference gene amplification curves



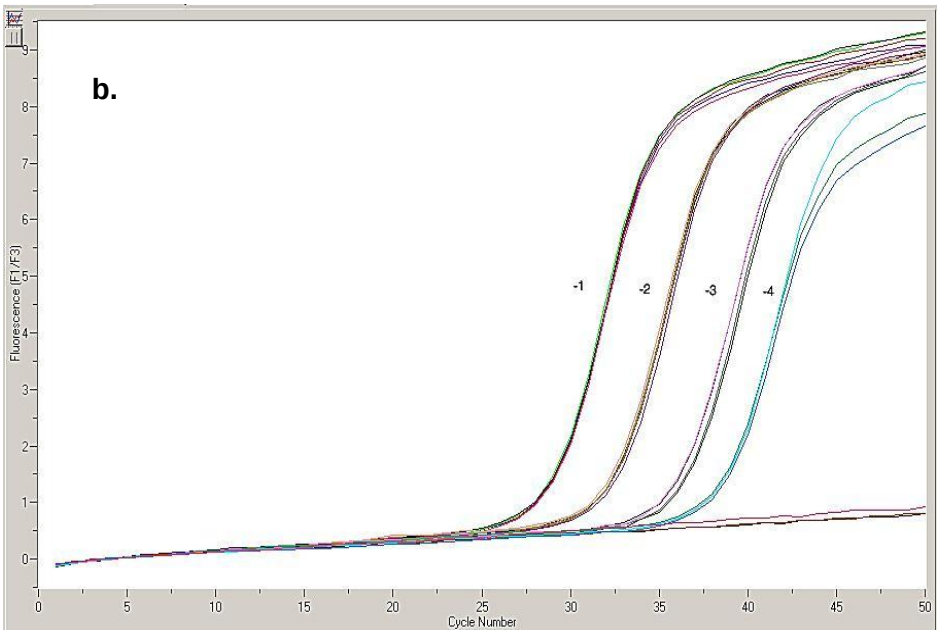


Table 13: a) Median CP values of the standard curve target gene experiment

Dilution of Kasumi-1 cDNA	Median CP value
1:10	25.3
1:100	28.81
1:1000	32.17
1:10000	35.195
1:50000	37.82

b) Median cp values of the reference curve target gene experiment

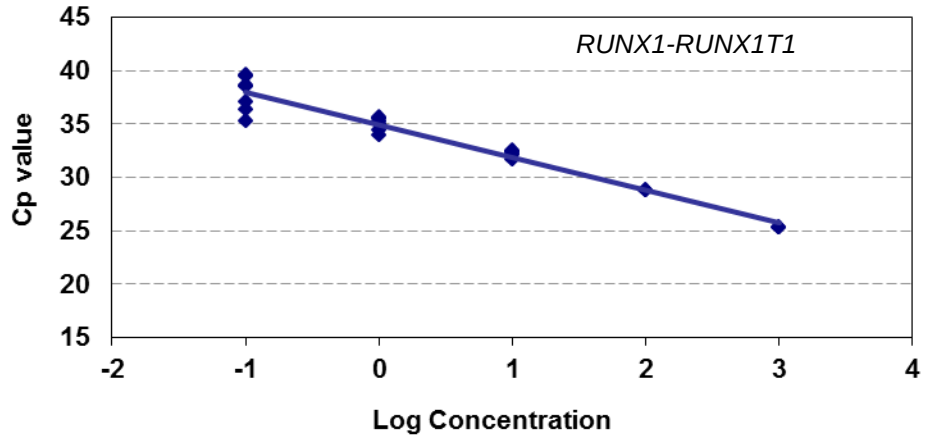
Dilution of Kasumi-1 cDNA	Median CP value
1:10	28.55
1:100	32.01
1:1000	35.36
1:10000	38.73

The CP differences between the average CP values of the 10fold dilutions theoretically should be 3.3 corresponding to a slope of the standard curve of -3.3 and to a maximum efficiency of 2.0 (van der Velden et al., 2003). CP values of target and reference curve are presented in Table 13.

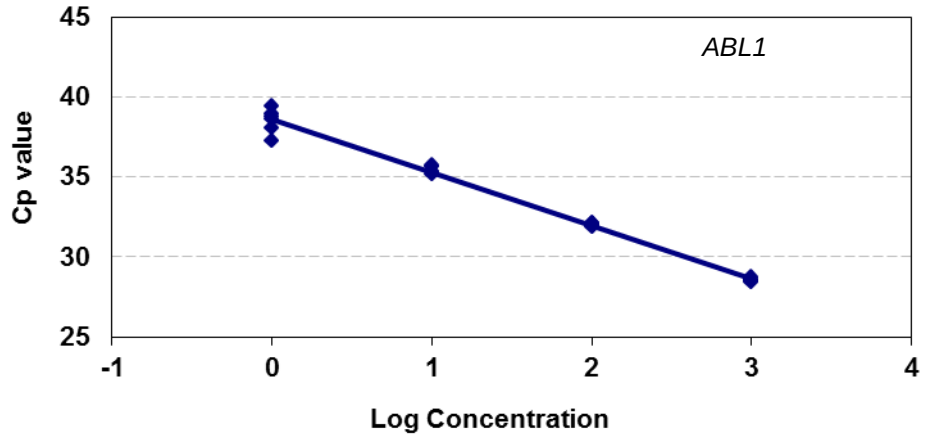
The efficiency (E) and the error (r) for the amplification of *RUNX1-RUNX1T1* was 1.999 and 0.0532 respectively (Fig 9a). For *ABL1* amplification the efficiency and error was 1.973 and 0.0171 (Fig 9b). According to the manufacturer's instructions (Roche Applied Science, Technical Note No LC 10/update 2003) the optimal efficiency is 2.0 and an error below 0.2 is acceptable (Fig 9).

Figure 9: Standard curves prepared from the data in figure 8 a) *RUNX1-RUNX1T1* standard curve: slope -3.3, E=1.999 and r= 0.0532. (b) *Abl1* standard curve: slope -3.4, E=1.973 and r=0.017

a)



b)



4.2.7 Reproducibility, sensitivity and specificity of the assay

To determine the sensitivity of the assay, serial dilutions of cDNA from Kasumi-1 cell line were used as a template in *RUNX1-RUNX1T1* and *ABL1* RQ-PCR. In order to check the reproducibility of the assay each experiment was repeated 5 times. The maximum sensitivity for both assays was higher than 10^{-4} ($5x10^{-4}$) (Table 14). Maximum sensitivity is defined as the lowest dilution step giving specific amplification. Additionally the experiment was repeated using a known concentration of cDNA from 3 *RUNX1-RUNX1T1* patients that was serially diluted (1:10-1:50000). The maximum sensitivity reached was higher than 10^{-4} ($5x10^{-4}$) (Table 15).

In order to determine the specificity of the assay, *RUNX1-RUNX1T1* negative patients were tested; along with cDNA from K562, ME1, NB4, OCI, and SD1 cell line. No amplification was observed (data not shown).

Table 14: Median CP values of 5 repeated experiments to check the reproducibility and sensitivity of the assays

a) Target gene

Kasumi-1 cDNA	Experiments I	II	III	IV	V
Serial dilutions	CP values (Median)	CP values (Median)	CP values (Median)	CP values (Median)	CP values (Median)
1:10	25.79	25.54	25.55	25.22	25.3
1:100	29.67	29.5	29.52	28.75	28.81
1:1000	33.01	33.11	33.15	32.03	32.17
1:10000	36.24	36.44	35.69	35.31	35.19
1:50000		40.09	39.32		37.82

b) Reference gene

Kasumi-1 cDNA	Experiments I	II	III	IV	V
Serial dilutions	CP values (Median)	CP values (Median)	CP values (Median)	CP values (Median)	CP values (Median)
1:10	29.26	28.68	28.91	28.71	28.55
1:100	32.40	32.01	32.22	32.11	32.01
1:1000	35.71	35.49	35.92	35.81	35.36
1:10000	38.74	39.19	38.9	38.52	38.73
1:50000	39.54		40.0		38.99

Table 15: Sensitivity of the assay using serial dilutions of known cDNA concentration from 3 *RUNX1-RUNX1T1* positive patients

	Patient 1	Patient 2	Patient 3
Serial dilutions of cDNA	CP values (Median)	CP values (Median)	CP values (Median)
Kasumi-1 1:10	24.85	25.27	25.52
1:10	26.57	24.10	25.88
1:100	30.04	31.40	29.36
1:1000	33.69	33.50	32.89
1:10000	36.19	35.09	35.75
1:50000	37.54	37.57	38.25

Additionally 30 patient samples at various disease stages were amplified with RQ-PCR and conventional PCR in order to further investigate the sensitivity and specificity of the assay. As shown in Table 16, 26 out of 30 samples were positive with RQ-PCR whereas only 24/30 were tested positive using 5 µl in the nested reaction. As expected 29/30 were positive using 10 µl in the nested reaction.

Table 16: Patient samples (N=30) at various disease stages

Patients	RQ-PCR	Primary RT-PCR	Nested RT- PCR	
	CP values (Median)		5 µL	10 µL
1	20.76	+	+	+
2	26.67	+	+	+
3	33.78	+	+	+
4	35.62	+	+	+
5	_	_	_	_
6	21.33	+	+	+
7	24.33	+	+	+
8	22.70	+	+	+
9	26.67	+	+	+
10	28.01	+	+	+
11	30.74	+	+	+
12	_	_	+	+
13	36.62	_	_	+
14	_	_	+	+
15	26.13	_	+	+
16	26.71	_	_	+
17	_	_	+	+
18	31.14	+	+	+
19	21.79	+	+	+
20	30.96	+	+	+
21	25.32	+	+	+
22	35.30	_	+	+
23	23.78	+	+	+
24	38.69	_	_	+
25	37.07	_	_	+
26	36.82	_	+	+
27	30.05	_	+	+
28	31.16	_	+	+
29	35.96	_	+	+
30	37.34	_	_	+
Positivity (%)	86.6%	50%	80%	96.6%

Thus the sensitivity of RQ-PCR was 100% when compared to RT-PCR with 1 μl of PCR product, 87.5 and 89.5% compared to RT-PCR using 5 and 10 μl of primary PCR product. The specificity of the assay was 26%, 16% and 100% respectively. (Table 17).

Having established the assay, a total of 184 samples (39 patients) in diagnosis and during follow up (paragraph 3.1.3, page 24) were tested in parallel with conventional nested PCR (paragraph 4.3).

Table 17*:* Sensitivity and specificity of RQ-PCR

"gold standard 1"

RT-PCR 1μl

Condition positive	*Condition negative*
True positive = 15	False positive = 11
False negative = 0	True negative = 4
Sensitivity = 15/15=1=100%	Specificity = 4/15=0.26=26%

"gold standard 2"

RT-PCR 5μl *

Condition positive	*Condition negative*
True positive = 21	False positive = 5
False negative = 3	True negative = 1
Sensitivity = 21/24=0.875=87.5%	Specificity = 1/6=0.166=16%

"gold standard 3"

RT-PCR 10μl *

Condition positive	*Condition negative*
True positive = 26	False positive = 0
False negative = 3	True negative = 1
Sensitivity = 26/29=0.895=89.5%	Specificity = 1/1=100%

*nested reaction

4.2.8 Evaluation of RQ-PCR data

4.2.8.1 Data transfer in LightCycler Software 4.05

Data were collected using the LightCycler Software 3.5 and were transferred into the LightCycler software 4.05 in order to analyze the data using the "Second Derivative maximum" method, where CPs are automatically identified and measured at the maximum acceleration of fluorescence. The above method was chosen instead of the "Fit Point method" as the second is influenced by the user, who has to determine the baseline adjustment, noice band setting, crossing line setting and choice of fit

points (Luu-The et al., 2005). “Second Derivative Maximum” is more precise in quantifying low gene expressions levels (Luu-The et al., 2005).

4.2.8.2 Quantification analysis

The principle of the relative quantification has already been explained in paragraph 3.3.2. Quantification analysis uses the CP value of each sample, to determine the relative concentration of the target gene compared to a calibrator. Relative quantification analysis was performed using the “Relative quantification Mono-Color” (single channel) settings. As it has already been mentioned, an optimized external standard curve was imported for the evaluation of each run. The calculation of the CP values was made using the “Automated method” according to the recommendations of the manufacturer.

4.3 Nested RT-PCR compared to RQ-PCR

The aim of this study was to establish the possible role of RQ-PCR for MRD monitoring in AML patients bearing the *RUNX1-RUNX1T1* fusion gene. The results were compared to nested RT-PCR which was performed in parallel as already mentioned. In total 184 samples were tested. Of these, 118 where positive and 66 where negative in RQ-PCR.

Interestingly, we observed a discrepancy in the results of RQ-PCR and nested RT-PCR. One sample (1/118) was positive only in RQ-PCR and negative in RT-PCR.

On the other hand, in the samples where no amplification was detected by RQ-PCR, 24/66 were positive by nested RT-PCR.

In cases where no amplification was observed in RT-PCR, more PCR template was used in the nested PCR reaction.

When 5 μl of PCR template in nested reaction, gave no amplification, the reaction was repeated using 10 μl of primary PCR product.

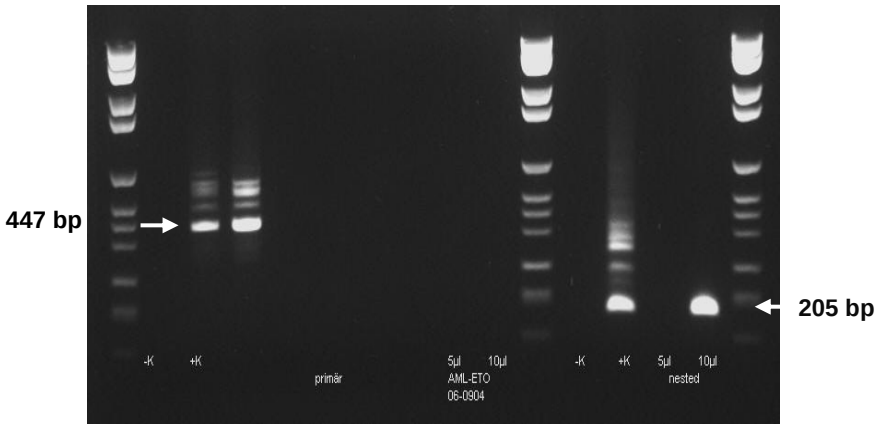
Unsurprisingly when 10 μL were used, the PCR sensitivity was rising and 5/24 samples became positive (Fig 10).

Figure 10: Quantification of *RUNX1-RUNX1T1* transcripts: a-1) quantification of *RUNX/RUNX1T1* for sample 06-0904, b-1) primary and nested PCR for the same sample, a-2) quantification of *RUNX/RUNX1T1* for the sample 06-0021, b-2) primary and nested PCR for the same sample

a-1)

Sample Type	Pos	Sample Name	Cp Median	Concentration Ratio	Normalized Ratio
Target Calibrator	1, 2	kasumi 1:10	25,07	7,51	1,00
Reference Calibrator	11, 12	kasumi 1:10	27,98		
Target Unknown	8	06-0904	37,26	[6,73E-4]	[8,96E-5]
Reference Unknown	18	06-0904	26,71		

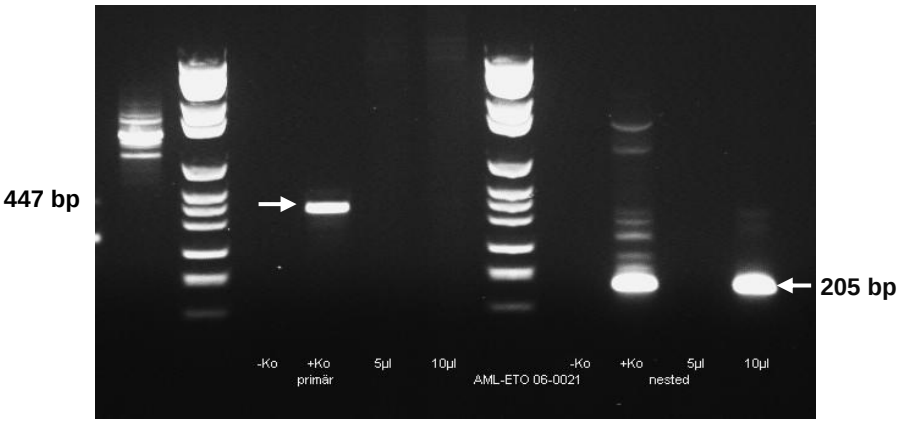
b-1)



a-2)

Sample Type	Pos	Sample Name	Cp Median	Concentration Ratio	Normalized Ratio
Target Calibrator	1, 2	kasumi 1:10	25,07	7,51	1,00
Reference Calibrator	11, 12	kasumi 1:10	27,98		
Target Unknown	5	06-0021	36,62	[6,11E-4]	[8,14E-5]
Reference Unknown	15	06-0021	25,93		

b-2)



4.4 Application of the optimized *RUNX1-RUNX1T1/ABL1* RQ-PCR protocol on t(8;21) positive AML patient samples

4.4.1 Patients' characteristics

39 *RUNX1-RUNX1T1* positive patients, that had been referred to the Laboratory for Leukemia Diagnostics, Klinikum Großhadern, were retrospectively investigated for routine cytogenetic and molecular analysis. Patients' characteristics, cytogenetics and additional genetic alterations are listed in Table 4, 5 and 6, respectively on page 25.

The frequency of *FLT3*-ITD and *FLT3*-D835 mutation was 5.5% (2/36) and 2.7% (1/36), respectively (Schnittger et al., 2003, Schnittger et al., 2004). The *KIT*-D816 mutation was found in 16.1% (5/31). None of the patients had the *MLL*-PTD mutation (Markova et al., 2009, Shen et al., 2011).

4.4.2 RQ-PCR

4.4.2.1 *RUNX1-RUNX1T1* transcript levels at diagnosis

29 patients were tested at diagnosis (28 BM samples and 1 PB). The *RUNX1-RUNX1T1* ratio ranged from 8.77 to 131.00 with a median of 29.37.

4.4.2.2 MRD monitoring

4.4.2.2.1 RUNX1-RUNX1T1 transcript levels at day 16 of the induction treatment

RUNX1-RUNX1T1 levels were measured on day 16. 12 out of 39 samples were available (BM=11, PB=1). The levels of *RUNX1-RUNX1T1* ratio ranged from 0.009 to 28.96 with a median value of 2.11. In 6 patients, the reduction of the *RUNX1-RUNX1T1* transcript levels between diagnosis and d16 was only 1 log. 3 out of these 6 patients relapsed. None of them had an early relapse (defined as relapse before 6 months).

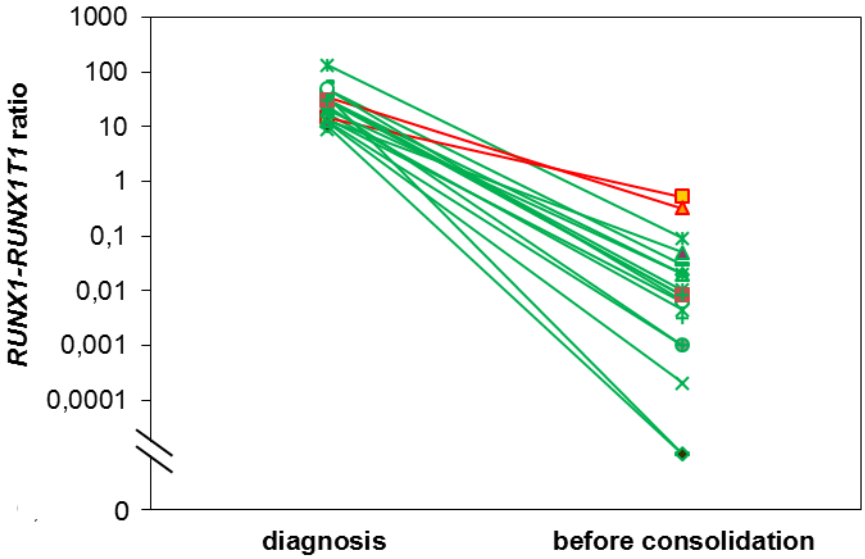
4.4.2.2.2 RUNX1-RUNX1T1 transcript levels before consolidation

23 patients were analyzed at about 2 months post diagnosis, before consolidation therapy (data not shown). The minimum value of *RUNX1-RUNX1T1* ratio was 0.00001 and the maximum was 0.52.

20 out of 23 patients had paired samples at diagnosis and before consolidation. A decrease of *RUNX1-RUNX1T1* expression between these two time points (3 logs) was observed in 18/20 patients and is presented in Fig 11 as green lines. Only three out of 18 patients (16.6%), with a 3-log reduction of *RUNX1-RUNX1T1* expression

levels, relapsed. However only 2 patients had a less than 3 log reduction (Fig 11 red lines). Interestingly one of these two patients relapsed, but the group size was not sufficient for statistical analysis or calculation of cutoff-values for the relapsed risk.

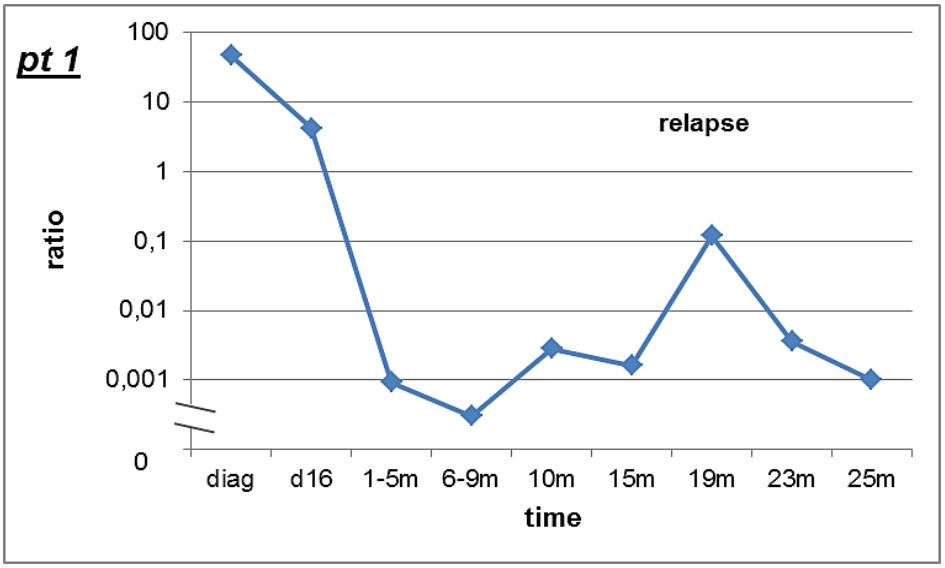
Figure 11: *RUNX1-RUNX1T1* transcript levels at diagnosis and before consolidation

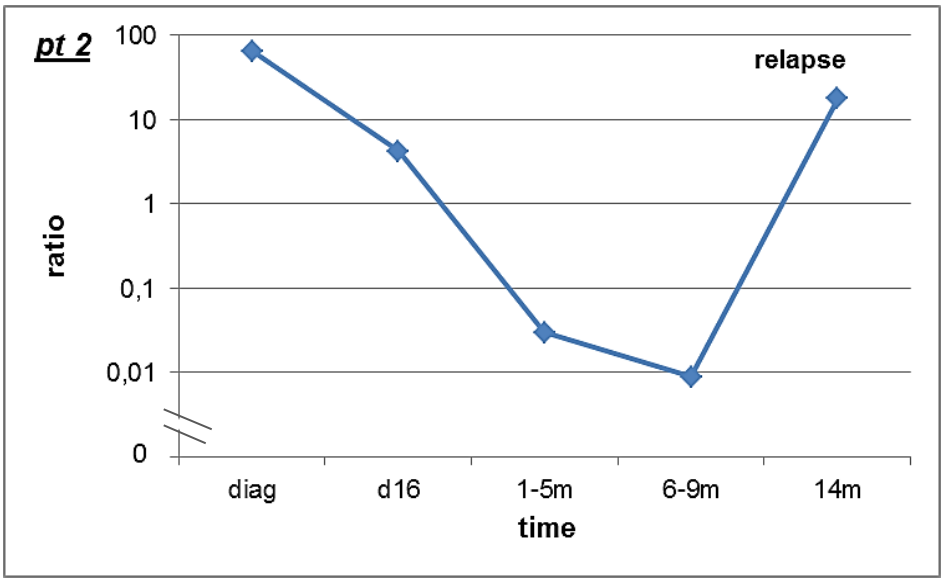


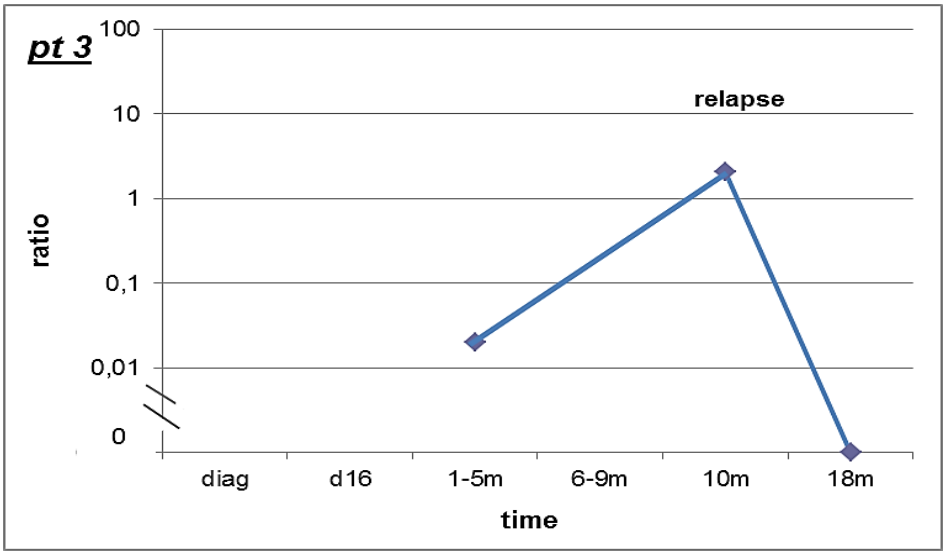
4.4.2.2.3 RUNX1-RUNX1T1 transcript levels at relapse

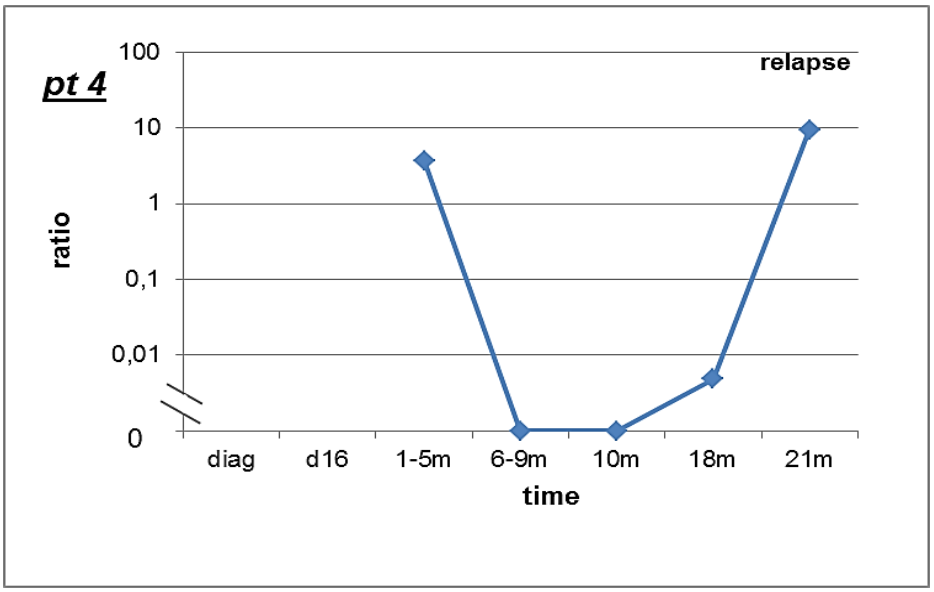
The median follow up of the 39 patients was 24 months (range 1.7-71). Despite the favorable prognosis of t(8;21), 28% (11/39) of the patients in the study relapsed and 5 of them, died. At relapse only 5 patient samples were available. Kinetics of MRD levels of the 5 relapsed patients are presented in Figure 12. The range of *RUNX1-RUNX1T1* transcripts was 0.12-35.19 (median 9.45). One of the patients (Fig12. pt 5) was found to be also positive for *KIT*-D816 mutation. Figure 13 (page 54), shows the *RUNX1-RUNX1T1* fusion transcript levels at diagnosis, d16 of the treatment, before consolidation and at relapse.

Figure 12: ratio levels of *RUNX1-RUNX1T1* at relapse in 5 patients








pt 4
relapse
100
10
1
0,1
0,01
0
ratio
diag
d16
1-5m
6-9m
10m
18m
21m
time

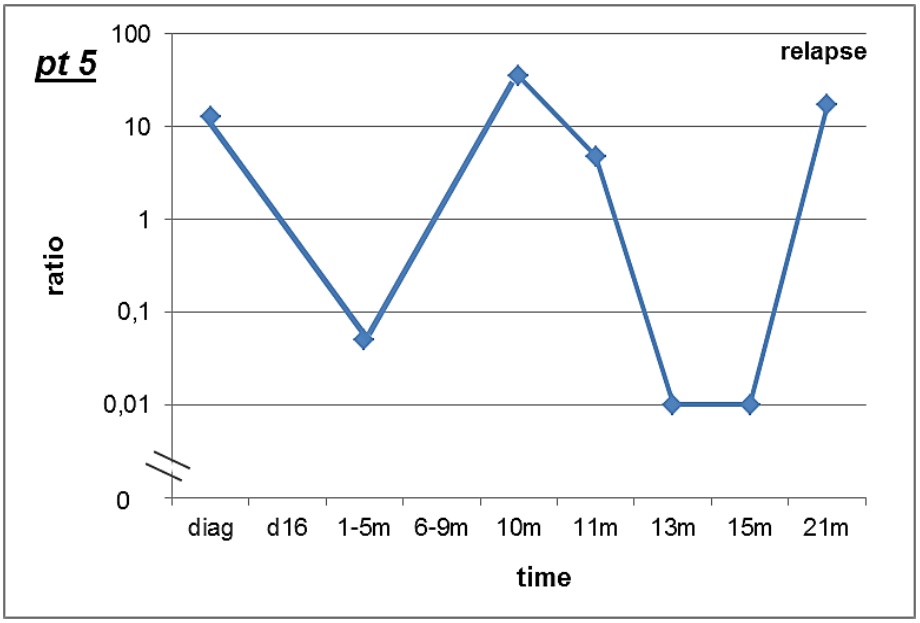
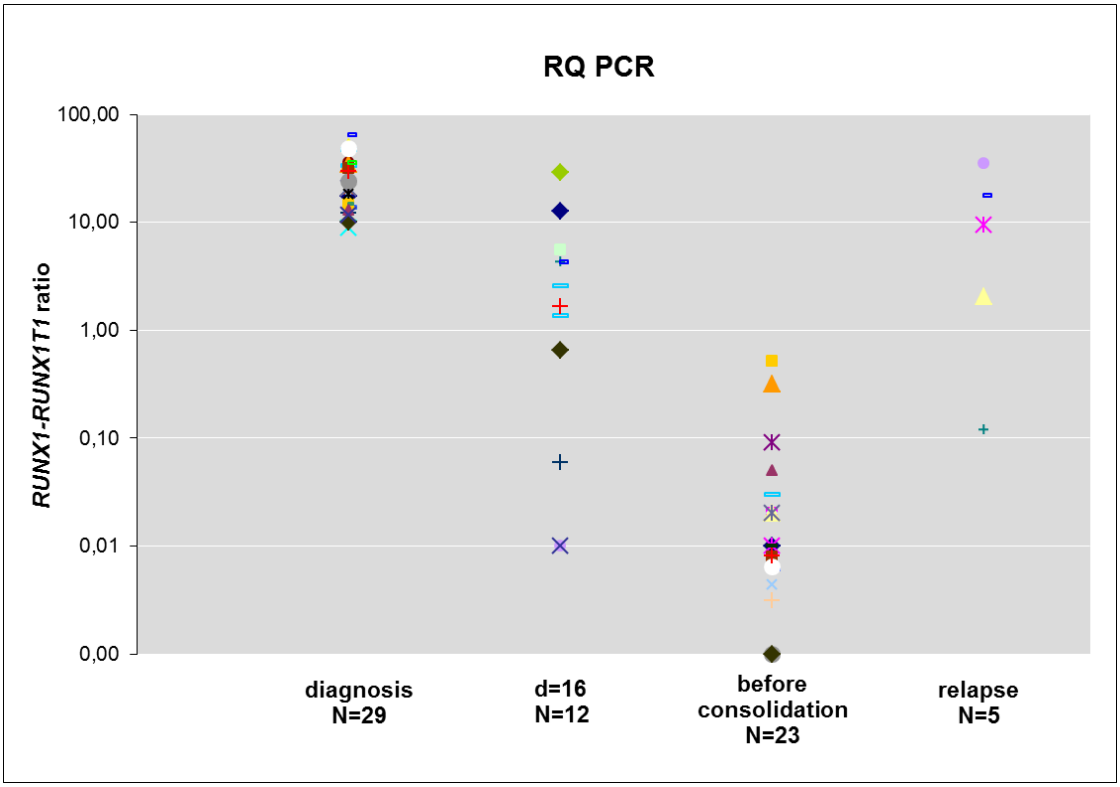

pt 5
relapse
100
10
1
0,1
0,01
0
ratio
diag
d16
1-5m
6-9m
10m
11m
13m
15m
21m
time

Figure 13: ***RUNX1-RUNX1T1*** **transcript levels at different stages of the disease (patients=39)**



4.4.3 Application of nested RT-PCR on patient samples

MRD monitoring of 18 patients, being in morphological CR after chemotherapy, is presented in Figure 14a. Six of these patients (pt 8, 9, 10, 13, 14 and pt 18) were nested PCR positive when checked one year after being in long term remission. Three out of these 6 patients (pt 9, 10, and pt. 18), had also detectable transcripts by RQ-PCR.

None of these 6 patients relapsed within a follow up time of 18.8, 12, 46.8, 36.3, 41.9 and 12 months, respectively.

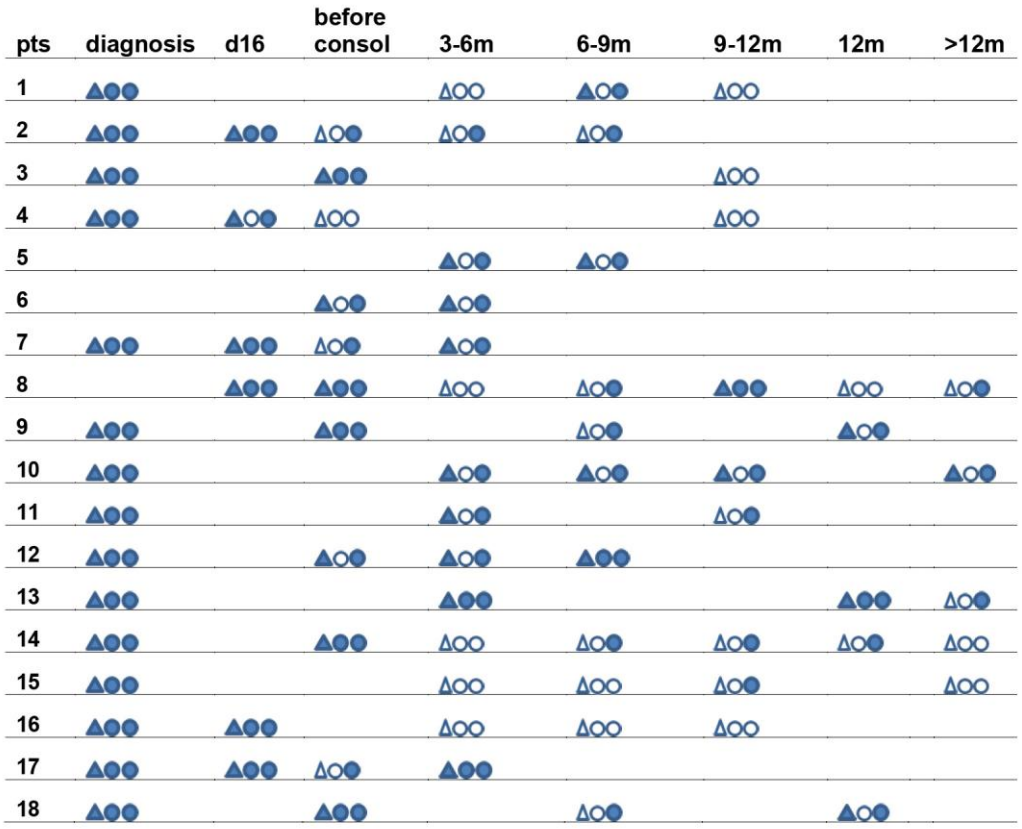
Although CBF leukemias, are considered favorable - risk diseases, additional mutations like *KIT,* as well as cytogenetic and clinical (e.g. chloroma) risk factors, may have negative impact on prognosis and therefore should be taken into consideration for patients suitable for BMT.

MRD monitoring of nine patients after BMT, 8 patients after allogeneic BMT and one patient after BMT (Fig 14b, pt. 8), is presented in Figure 14b. In this cohort of 9 patients, 4 were nested PCR positive for *RUNX1-RUNX1T1* after BMT. Only one of them, (Fig 14b, pt. 4), became RQ-PCR positive 9 months after BMT, and relapsed 5 months later. It is of notice, that a second patient (Fig 14b, pt. 5), being RQ- and

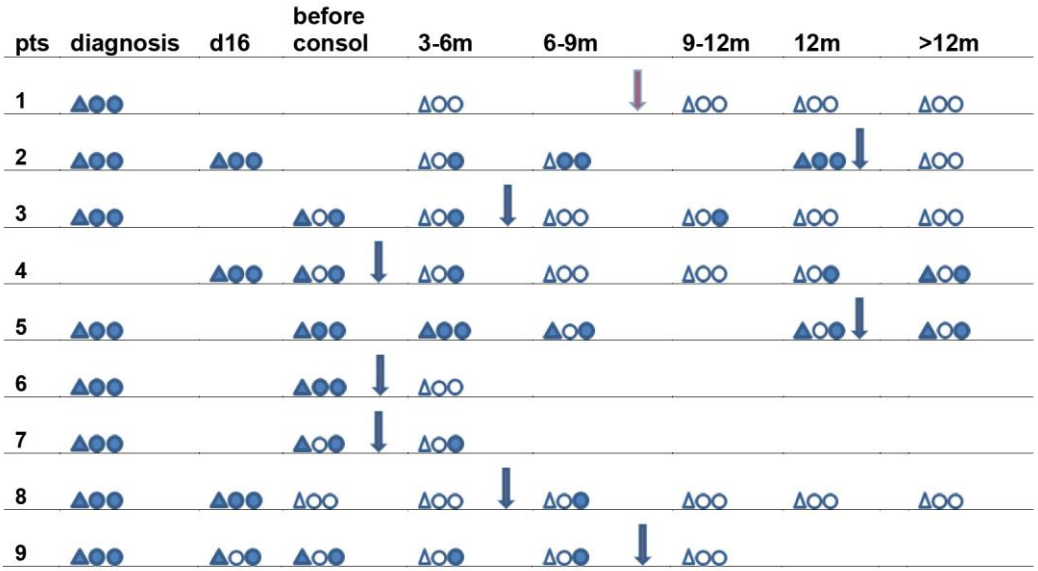
nested PCR positive post BMT, became negative seven months later using both methods.

Figure 14: Comparison of RQ-PCR with RT-PCR at various check points. The *blank triangle* represents RQ-PCR negative samples, and the *black* RQ-PCR positive samples. The first cycle represents the primary PCR and the second cycle the nested PCR. *Blank cycle* indicates negative PCR and *black cycles positive* PCR. Arrows indicate BMT.

a) patients in remission after chemotherapy



b) patients received BMT



4.5 *RUNX1-RUNX1T1* in *JAK2* positive myeloproliferative neoplasms

In 2007, while the study was in progress a very interesting case report was studied at the Laboratory for Leukemia Diagnostics (Schneider et al., 2007). A 60 year old female patient was diagnosed in April 2004 with Polycythemia Vera. She had a normal karyotype and she was homozygous for *JAK2*V617F. Since then, she was treated by phlebotomy until February 2006 and then with hydroxyourea (HU). In July 2007, despite the use of HU (for only 6 months), she developed leukocytosis and underwent new cytogenetic analysis which revealed an additional t(8;21). The result was confirmed by FISH analysis and RT-PCR. Although the role of HU in leukemogenesis cannot be ruled out it seems less probable due to the short use of it.

This study provided for the first time a clear clinical evidence for the possible timely acquisitions of *JAK2*V617F mutation and *RUNX1-RUNX1T1* fusion gene in a patient.

As it is described in paragraph 1.2 (page 11), according to the “two-hit model” hypothesis of AML, *RUNX1-RUNX1T1* and *JAK2*V617F may coexist, since both mutations may cooperate in leukemogenesis.

It is known that *JAK2*V617F mutation is rare in *de novo* AML and little is known about the frequency of this mutation in specific cytogenetic risk groups of AML and about its impact on prognosis.

To clarify the incidence and the prognostic significance of this additional mutation in our cohort, 18 patients were screened (Table 6, page 25) using melting curve analysis. No *JAK2*V617F mutation was detected.

5. DISCUSSION

5.1 *RUNX1-RUNX1T1* RQ-PCR

Nowadays, Real-time Quantitative RT-PCR (RQ-PCR) is the most popular method for the quantification of RNA in biological samples (Huggett et al., 2005). Furthermore, the method is highly sensitive and thus most suitable for MRD detection (Beillard et al., 2003). By performing a parallel amplification of a reference gene, known as housekeeping gene or endogenous control gene (Beillard et al., 2003), the RNA quality and quantity can be evaluated using the same technology.

In order to supplement and verify molecular and standard cytogenetic diagnostics of *RUNX1-RUNX1T1* fusion gene detection, our aim was to establish a fast and sensitive assay to detect and quantify *RUNX1-RUNX1T1* transcripts at diagnosis and during follow-up. Thus, in contrast to nested PCR, a quantification of low transcript levels should be achieved. In this work, a quick (results in less than 1 hour), stable and accurate Taqman based RQ-PCR assay for *RUNX1-RUNX1T1* and *ABL1* housekeeping gene was established and validated using the LightCycler platform and has been integrated in the routine diagnostic and follow-up of AML patients with t(8;21). Using this technology, the PCR amplification occurs in special closed glass capillaries, therefore the risk of contamination is very low.

5.1.1 Choice of Reference gene

To establish an accurate assay for quantitative MRD (RQ-PCR), it is of major importance to correct for sample to sample variations, variations in the integrity of RNA, differences due to RT-PCR efficiency and cDNA sample loading (Stahlberg et al., 2003, Stahlberg et al., 2004). Therefore, a housekeeping gene should be amplified in parallel to the target gene, ideally being optimized to run at the same conditions in the same run. This gene should be located on a chromosome which is not often involved in chromosome loss and gain, it should be expressed at similar levels in different cell types and it shouldn't be connected to the cell cycle or to the cell activation (van der Velden et al., 2003).

Within the Europe Against Cancer Program (Gabert et al., 2003), various genes were candidates for control genes. *ABL1* (GenBank Accession Number M14752), was recommended as the most suitable gene for this purpose, since its expression is constant between BM and PB samples as well as between normal and leukemic

samples and shows no pseudogenes (van der Velden et al., 2003). *ABL1* is located on the long chromosome of chromosome 9 (9q34) (Beillard et al., 2003). In this study, the PCR assay was optimized so that the amplification of the *ABL1* reference gene and *RUNX1-RUNX1T1* target gene had a similar efficiency and sensitivity as it is shown in Figure 8 (page 42) and 9 (page 44). The efficiency for the *RUNX1-RUNX1T1* amplification was 1.999 and for the *ABL1* amplification the efficiency was 1.973.

5.1.2 Normalization of the assay

RQ-PCR is a method that offers great accuracy in the quantification of the PCR product and is characterized by its high sensitivity. To achieve this, it is necessary to apply normalization strategies to the RQ-PCR data (Huggett et al., 2005). For this study, the first step was the normalization of the number of cells in the patient samples. Thus after isolation of PB mononuclear cells, cell counting was performed using the Microcell counter. Aliquots of $10x10^6$ cells from samples at diagnosis and follow up respectively were used for RNA extraction. To ensure accurate quantification and qualification of RNA before reverse transcription, the amount of cells in RLT buffer, used to create cDNA, was the same for all diagnosis samples as well as for all the follow-up samples. This way, there was no need to perform normalization with RNA quantification before reverse transcription, as it is described by Hugget *et al* (Huggett et al., 2005). Possible degradation during storage of the lyzed cells or during RNA of cDNA synthesis steps needs to be normalized by the use of a reference gene.

Some authors (Huggett et al., 2005) suggest the use of multiple reference genes, which is however often difficult to perform in routine diagnostics due to limited sample material and higher costs. Finally, normalization between different runs was achieved with the use of a calibrator. The *RUNX1-RUNX1T1/ABL1* expression ratios in all samples were divided by the *RUNX1-RUNX1T1/ABL1* expression ratio of a calibrator (paragraph 4.2.2, page 40). Therefore, the ratio of target to reference gene for the calibrator should be constant.

Target and reference genes showed stable expression in the cDNA from Kasumi-1 cell line (that was used as calibrator), allowing the correction of variations in detection sensitivity due to sample quality between every run.

5.1.3 Sensitivity and reproducibility

Maximum sensitivity is defined as the lowest dilution giving specific amplification**.** Sensitivity is affected by the number of cells examined, by the amount of RNA/cDNA that is analysed and by the number of PCR cycles (van der Velden et al., 2003).

RQ-PCR assay for MRD analysis should reach a sensitivity of min 10^{-3} up to 10^{-4} and 10^{-5} (van der Velden et al., 2003). Assays that target sequences such *as FLT3*-ITD and *WT-1* reach sensitivities of at least 10^{-4} (van der Velden et al., 2003). Maximum sensitivity of 10^{-5} is observed in assays targeting a *NPM1* mutation (Papadaki et al., 2009).

In this study the maximum sensitivity of the assays for both target and reference gene, was more than 10^{-4} ($5x10^{-4}$) and found to be in accordance with Gabert J., et *al* (Gabert et al., 2003). The sensitivity of $5x10^{-4}$ was also confirmed using cDNA dilutions from Kasumi-1 cell line as well as dilutions (1:10-1:50000) of cDNA with known concentration from *RUNX1-RUNX1T1* patients. Additionally 30 patient samples at various disease stages were amplified with RQ-PCR in order to further investigate the sensitivity of the assay (Table 16, page 46).

In the study of MRD detection by van der Velden *et al*, (van der Velden et al., 2003) it is clear that the standard curve obtained by the 10 fold dilutions, should have a slope of -3.3. Thus when the logarithmic value of the dilution is plotted against the CP value, a standard curve with a slope of the of -3.3 is created and corresponds to maximum efficiency of 2.0. In practice slopes between -3.0 and -3.9 are acceptable (van der Velden et al., 2003). In this assay, the slope of the standard curve of the target gene was -3.32 and for the reference gene was -3.48, showing a good linearity and reproducibility. Standard curves are presented in Figure 9 a and b, page 44.

5.2 Comparison of RQ-PCR to qualitative PCR

The results of the quantitative PCR were in accordance with the results of the primary PCR, but the sensitivity of RQ-PCR was lower, compared to the nested PCR, since 24 samples were negative by RQ-PCR but positive by nested RT-PCR, as it is shown in Figure 14a and b, page 55 and 56.

6 patients were positive in nested PCR although they were in a long term remission ≥12 months. Interestingly in one patient who underwent BMT, we observed the same discrepancy in the results of quantitative and qualitative PCR. It is of notice that this patient had a morphological relapse 5 months later.

Several studies in the past have described the persistence of *RUNX1-RUNX1T1* transcripts by nested RT-PCR, in patients being in long term remission, even after BMT.

Consequently, if the sensitivity of the RQ-PCR is very high, detection of MRD can be troubled.

Miyamoto *et al* (Miyamoto et al., 1996), examined 22 patients with RT-PCR. 18 patients were positive although they were in remission for 12-150 months. 4 of the patients had undergone allogeneic BMT. These positive results were attributed to the presence of residual multipotent hematopoietic progenitors retaining the t(8;21).

Saunders *et al* (Saunders et al., 1994), reported 6 patients (in a cohort of 7), studied with RT-PCR, to be positive for the chimeric gene *RUNX1-RUNX1T1* while being in remission. 2 patients were transplanted (1 patient with allogeneic BMT and the other with autologous BMT).

Jurlander *et al* (Jurlander et al., 1996) presented the detection of *AML-ETO* transcripts with RT-PCR in 9 of 10 patients in CR after BMT, suggesting that the fusion transcript itself is not capable of inducing malignant transformation.

Zhang *et al* (Zhang et al., 2013), recently screened, BM samples from 52 patients. 32 patients were examined using nested RT-PCR (qualitative evaluation) and 20 patients were examined with RQ-PCR (quantitative evaluation). Interestingly, the authors report that the results of the nested RT-PCR were of great prognostic value in the following time intervals: 6-12 months and 12-18 months. Patients with negative *RUNX1-RUNX1T1* transcripts and duration of CR>1 year had low probability to relapse. They concluded that continuous negative results with RT-PCR indicate low risk of relapse.

However, the number of patients included in these reports was small.

5.3 Impact of prognostic factors in disease progression

Despite the fact that AML with t(8;21) has a favorable prognosis, 28% of patients in this study relapsed, a result that is in accordance with the literature (Yin et al., 2012, Leroy et al., 2005, Schnittger et al., 2003).

Among the ‘’classical’’ adverse prognostic factors for the disease, are the high WBC, as well as the age at diagnosis, as observed by a number of researchers.

For the relapsed patients in the study, the median WBC was $7x10^3$/μL (min $2.6x10^3$/μL- max $43x10^3$/μL) and the median age at diagnosis was 52.2 years (range: 15,8-63,5).

Nguyen *et al* (Nguyen et al., 2002) analyzed 161 patients of the French AML Intergroup, and observed that patients with low WBC index (WBC ratio to marrow blast), had a better prognosis.

Martin *et a* (Martin et al., 2000), commented that WBC count had a significant prognostic influence on DFS.

Recently Hoyos *et al* (Hoyos et al., 2013) reported a cut-off value of $20x10^3$/μL in WBC at diagnosis, as a prognostic factor. They also comment that for patients above the age of 50, the current therapeutic protocols are not sufficient enough.

As it is presented in Table 6 (page 25), *KIT*-D816 mutation was detected in 5/36 patients (13.8%) in this study. Patients harboring the mutation presented at diagnosis with median WBC $13.3x10^3$/μL (min $6.9x10^3$/μL-max $31.7x10^3$/μL). Although the sample of our study is small, the presence of *KIT*-D816 mutation was observed in 1 of the relapsed patients. Additionally from the 39 patients participating in the study *FLT3*-ITD mutation was observed in two and *FLT3*-D835 in one patient (Table 6).

KIT-D816 mutation has been recently recognized as an adverse prognostic factor in *RUNX1-RUNX1T1* positive AML (Dohner and Dohner, 2008, Schnittger et al., 2006b).

Schnittger *et al* (Schnittger et al., 2006b), reported the presence, as well as the unfavorable impact in prognosis of the *KIT*-D816 mutation, in 24,2% of *RUNX1-RUNX1T1* positive patients, from a cohort of 1940 randomly selected AML patients. The authors suggested that patients harboring this mutation are candidates for more intense or targeted treatments with tyrosine kinase inhibitors. In the same study however, *FLT3* mutations were rarely detected in this subgroup of AML.

The adverse prognostic significance of *KIT*-D816 mutation in CBF leukemias was also suggested by two other studies. In the first study, Cairoli *et al* (Cairoli et al., 2006) analyzed 67 adults. They found an incidence of 45% (19/42) among the patients with t(8;21) which was associated with higher white blood cell count at diagnosis, higher incidence of extramedullary leukemia, higher incidence of relapse and inferior OS. In the second study 121 Korean patients were retrospectively evaluated by Kim *et al* (Kim et al., 2013). Here the incidence of the mutation was lower 27% (22/82) in patients with t(8;21), however the negative predictive value in event-free survival (EFS) and OS was maintained.

This is in contrast with the experience of Riera *et al* (Riera et al., 2013) and Jones *et al* (Jones et al., 2010) who did not detect any difference in progress-free survival

(PFS) and OS, among CBF AML patients with additional *KIT*-D816 mutation. The frequency of the mutation found was 30.4% and 20% respectively.

Very recently Krauth *et al* (Krauth et al., 2014), analyzed 139 patients with t(8;21) positive AML, for mutations in *ASXL1, FLT3, KIT, NPM1, MLL, IDH1, IDH2, KRAS, NRAS, CBL and JAK2.* Notably 69/139 patients (49.6%), had an additional molecular mutation. *KIT* mutations were the most common, found in 16.5% of the patients and had adverse prognostic impact in OS. Mutations of the *ASXL1* were found in 11.5% of the patients and had adverse prognostic impact in EFS. *JAK2* was detected in 4/139 patients (2.9%) and *FLT3*-ITD in 7/9 patients (5%).

In 2013 Opatz *et al* (Opatz et al., 2013), reported for the first time, the presence of *FLT3*-N676K mutation in CBF leukemias at diagnosis, without concurrent *FLT3*-ITD mutation. The mutation was detected in one patient with t(8;21) 1/36 (3%) and in 5/84 (6%) patients with *CBFB-MYH11.* Although the number of *FLT3*-N676K positive patients is small, and the prognostic significance of the mutation has to be further evaluated, the authors report a tendency for lower CR rates in these patients.

5.4 Monitoring MRD in AML with t(8;21) by RQ-PCR

A lot of effort is being made to identify prognostic risk factors and evaluate the prognostic value for MRD monitoring.

RQ-PCR is a well-established method with a wide range of applications in medicine and it is used routinely for the quantification of hybrid genes, as for *PML-RARA* resulting from the translocation t(15;17). Therefore the development of sensitive and specific RQ-PCR assays suitable for routine MRD monitoring, such as quantitative PCR for *NPM1*A (the most common mutation in the *NPM1* gene) (Papadaki et al., 2009), will help identify the group of patients in higher risk of relapse and based on that will help to make therapeutic interventions. MRD directed therapy at the moment is restricted to APL (Grimwade et al., 2009).

The persistence of *RUNX1-RUNX1T1* fusion transcripts in patients being in long term remission, brings up many questions regarding the prognostic significance of *RUNX1-RUNX1T1* transcript quantification and the use of this method for MRD assessment.

It is well known that the amount of residual leukemic blasts in the BM, one week after the end of the induction therapy (Kern et al., 2003) is of major prognostic significance, since it reflects the chemosensitivity of the disease. Thus, in our study transcript

levels were measured on day 16 and were correlated with the disease outcome. The median value was 2.11 (range 0.009-28.96). A reduction of only one log was observed in 6 patients and three of them relapsed. None had an early relapse.

At about 2 months post diagnosis, before consolidation therapy, 23 patients were analyzed. Comparison of the levels at diagnosis and before consolidation, on 20 out of the 23 patients (as shown in Figure 11, page 51) revealed a statistically significant decrease of 3 logs, in the median value of *RUNX1-RUNX1T1* ratios, in 18/20 patients. Only three patients with such a pronounced decrease relapsed.

Tobal *et al* (Tobal et al., 2000) studied 25 patients with t(8;21) and found that a 2 to 3 logs reduction of the *RUNX1-RUNX1T1* fusion transcripts after induction treatment followed by another reduction for 2 to 3 logs after consolidation therapy, is of major clinical importance. Thus, regular monitoring of patients will help to early identify patients being at risk for relapse. The same investigators also reported an early intervention to prevent relapse based on MRD levels, in one patient after allogeneic BMT. The patient presented with increasing *RUNX1-RUNX1T1* transcript levels 22 months after transplantation, suggesting imminent hematologic relapse. Thus the authors decided to stop immunosuppressive treatment, resulting in acute Graft-versus-host disease (GVHD) leading to complete clinical remission.

Schnittger *et al* (Schnittger et al., 2003) studied 349 patients positive for *RUNX1-RUNX1T1*, *CBFB-MYH11* and *PML-RARA*, and evaluated the prognostic significance of quantifying fusion transcript levels using RQ-PCR. They conclude that the transcript levels at diagnosis as well as their log reduction <3, after consolidation identifies the patients being at high risk of relapse.

Weisser *et al* (Weisser et al., 2007) investigated the outcome of 45 t(8;21) positive *RUNX1-RUNX1T1* patients investigating the reduction of *RUNX1-RUNX1T1* levels after induction and consolidation therapies. In this study the *RUNX1-RUNX1T1* levels at the diagnosis did not influence the molecular response. Instead a reduction of 4 logs of the transcript levels after induction and consolidation therapy was of major prognostic significance. Thus the importance of *RUNX1-RUNX1T1* transcript levels at diagnosis is controversial since as shown in our assay too, the *RUNX1-RUNX1T1* transcript levels at diagnosis do not correlate with the clinical outcome of the patients. At diagnosis the levels of the transcripts for the patients who remained in CR had a median value 66.1 (range 8.77-131) and those for the relapsed patients had a median value 54.9 (range 12.84-65.41).

In 2012, within the MRC AML-15 trial, Liu Yin *et al* (Yin et al., 2012), reported that the presence of low levels of *RUNX1-RUNX1T1* copies in BM or PB at diagnosis correlate with a complete disease remission. Besides that, the increment of MRD

levels during follow up, predicts clinical relapse. The most important prognostic factor to predict relapse, is a log reduction of the transcripts in the bonne marrow less than 3 (<3) after induction treatment. The study evaluated 163 patients with t(8;21).
Recently, the results of the CBF-2006 trial (Jourdan et al., 2013) were published. 198 patients with CBF leukemias were randomized between an induction treatment and a reinforced one, with the consolidation courses being the same for both groups. The MRD response after the first consolidation (3-log reduction and the absolute levels of 0.1%) was the major prognostic factor of relapse. Specific mutations in the *KIT* and the *FLT3* genes and a high WBC count at diagnosis were shown to be relatively weak prognostic factors for relapse prediction.
Zhu *et al (Zhu et al., 2013)*, report that MRD-directed risk stratification treatment may improve the prognosis of patients with t(8;21). They analysed 116 patients and they suggested that log reduction of *RUNX1-RUNX1T1* transcripts <3 after the second consolidation, identifies patients belonging to the high risk group.
In conclusion, our study supports the importance of MRD monitoring with *RUNX1-RUNX1T1* fusion gene. We were able to show that clinical outcome is related to the transcript levels measured on day 16, and to the log reduction (3-logs) measured between diagnosis and before consolidation therapy (paragraph 4.4.2.2.2, page 50). However the importance of transcript levels at diagnosis itself requires further confirmation.

5.5 *RUNX1-RUNX1T1* and *JAK2*V617F mutation

The case report that was studied at the Laboratory for Leukemia Diagnostics in 2007 (Schneider et al., 2007), provided for the first time a clear clinical evidence for the two hit model of leukemogenesis, in a leukemia patient with preexisting MPN.
Here, we screened 18 t(8;21) positive patients for the presence of an additional *JAK2*V617F mutation, using melting curve analysis. We found no additional *JAK2*V617F in our small cohort (Table 6, page 25). This might be explained by the fact that all investigated patients had *de novo* AML.
It is well established that in about 0.5-6% of *RUNX1-RUNX1T1* positive patients, *JAK2*V617F point mutation is detected (Dohner et al., 2006, Steensma et al., 2006, Steensma et al., 2005).
Vicente et al (Vicente et al., 2007), screened 339 AML patients at diagnosis and found 3.2% frequency of the mutation. Interestingly a correlation of the mutation with

less differentiated leukemias is mentioned, since all the positive patients were classified as M1 and M2 according to FAB.

Schnittger *et al* (Schnittger et al., 2007b) screened 1372 patients with various chronic myeloproliferative disorders (CMPD). 178 patients had *de novo* AML and 11 of them were positive for the mutation. However, this cohort includes all the cytogenetic groups. From the 39 patients with therapy related AML (t-AML), 2 were found to be positive for the *JAK2*V617F.

Schnittger *et al* (Schnittger et al., 2007a) further screened 24 patients positive for t(8;21), of which 20 patients had *de novo* AML, 1 patient had *de novo* AML in relapse and 3 patients had t-AML. Interestingly, 2 of the 3 patients with t-AML additionally had a *JAK2*V617F mutation, suggesting that *JAK2*V617F might be an additional aberration in t-AML.

Further results on similar cases might help to clarify a risk for additional leukemogenic events associated with certain therapies.

6. SUMMARY

Acute myeloid leukemia (AML) with t(8;21) rearrangement, constitutes about 5% of all AML cases and is characterized by the presence of *RUNX1-RUNX1T1* fusion gene. Although this subtype, referred as CBF leukemia, belongs to the favorable cytogenetic risk group, 25% to 30% of the patients relapse. Detection of minimal residual disease (MRD) is of major importance since it evaluates the "depth" of the remission and therefore, risk adapted therapy based on early detection of relapse becomes feasible. *RUNX1-RUNX1T1* fusion gene could be used for MRD detection using RQ-PCR.

Within this work, a sensitive, specific and easy to perform, calibrator normalized relative quantification with external standards assay for *RUNX1-RUNX1T1* transcript levels, was established and has consequently been integrated in the routine diagnostic work-up of *RUNX1-RUNX1T1* positive patients. *ABL1* was used as a reference gene and cDNA of the Kasumi-1 cell line as a calibrator. Relative quantification was performed by calculating the *RUNX1-RUNX1T1/ ABL1* ratio, which was further normalized to the *RUNX1-RUNX1T1/ABL1* expression ratio of Kasumi-1 calibrator. Maximum sensitivity of the assays for both target and reference gene, was higher than 10^{-4} ($5x10^{-4}$). The sensitivity of $5x10^{-4}$ was also confirmed using cDNA dilutions from Kasumi-1 cell line as well as dilutions of patients' cDNA. The specificity of the assay was determined when *RUNX1-RUNX1T1* negative patients were tested; along with cDNA from negative cell lines and no amplification was observed.

184 samples from 39 patients, were quantified in order to assess the clinical usefulness of the assay. Transcript levels in 12 patients were measured on day 16 and were correlated to the clinical outcome. Moreover in 20 patients, comparison of the *RUNX1-RUNX1T1* ratios at diagnosis and before consolidation revealed a 3 $\log_{10}$ decrease, which was also correlated to the disease outcome. 28% (11/39) of the patients in the study relapsed, consistent with previous studies.

Very importantly, due to the good correlation of *RUNX1-RUNX1T1* transcripts with the disease course, this assay will also be used to monitor MRD of t(8;21) positive patients within the recently launched AMLCG2014 study.

7. Zusammenfassung

Die akute myeloische Leukämie (AML) mit t(8;21) macht ca. 5% aller AML Fälle aus und wird von der Präsenz des *RUNX1-RUNX1T1* Fusionsgenes charakterisiert. Obwohl dieser Untertyp, der als CBF Leukämie bezeichnet ist, zu den günstigen cytogenetischen Riskgruppen gehört, erleiden 25% bis 30% der Patienten ein Rezidiv. Die Detektion der minimalen Resterkranknung (MRD) ist somit von großer Wichtigkeit, denn es erlaubt die Tiefe der Remission zu überprüfen.

Eine risikoadaptierte Behandlung, die auf die frühe Detektion eines Rezidivs abzielt, ist mittels MRD konzeptionell machbar und sollte aus diesem Grund untersucht werden. Das AML-spezifische *RUNX1-RUNX1T1* Fusionsgen kann mittels RQ-PCR für die MRD Detektion benutzt werden.

In dieser Arbeit wurde eine sensitive, spezifische und leicht durchzuführende, mittels Kalibrator normalisierte, relative Quantifizierung für das *RUNX1-RUNX1T1* Transkriptionstadium etabliert. Diese wurde im weiteren in der Diagnostik bei *RUNX1-RUNX1T1* positiven Patienten benutzt. *ABL1* wurde als Referenzgen und cDNA der Kasumi-1 Zelllinie als Kalibrator benutzt. Die relative Quantifizierung wurde mittels der *RUNX1-RUNX1T1/ ABL1* Rate berechnet, welche weiterhin zur *RUNX1-RUNX1T1/ABL1* Expressionrate der Kasumi-1 Zellinie (Kalibrator) normalisiert wurde. Die maximale Sensitivität der Assays für beide Ziel- und Referenzgen war $5x10^{-4}$. Eine Sensitivität von $5x10^{-4}$ wurde sowohl mittels cDNA Verdünnungen von Kasumi-1 Zelllinien, als auch von Verdünnungen des Patienten cDNA bestätigt.

In 184 Proben von 39 Patienten wurde die Expression von *RUNX1-RUNX1T1* quantifiziert, um die klinische Nutzbarkeit des assays zu beurteilen. Die Expression wurde am Tag 16 nach Chemotherapie bei 12 Patienten gemessen und mit dem klinischen Ergebniss korreliert. Weiterhin wurde die *RUNX1-RUNX1T1 Expression* bei Erstdiagnose und vor Konsolidierung bei 20 Patienten bestimmt. Eine 3 $\log_{10}$ Abnahme war mit einer geringen Rezidivwahrscheinlichkeit assoziiert. Insgesamt erlitten 28% (11/39) der Patienten in der Studie ein Rezidiv, was mit vorhergehenden Studien vereinbar ist.

Die *RUNX1-RUNX1T1* Expression kann mittels RQ-PCR monitorisiert werden und wird zur Quantifizierung der MRD bei t(8;21)-positiven-Patienten innerhalb der geplanten multizentrischen AMLCG2014 Studie verwendet werden.

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10. ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
BM	bone marrow
BMT	bone marrow transplantation
bZIP	basic region leucine zipper
CBF	core binding factor
cDNA	complementary DNA
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
CML	chronic myelogenous leukemia
CPp	Crossing Point
CR	complete remission
DFS	disease- free survival
dNTPs	deoxynucleotide triphosphates
dsDNA	double-stranded DNA
EFS	event-free survival
ET	essential thrombocythemia
ETO	Eight-Twenty-One
EVI1	Ecotropic viral integration-1
FAB	French - American – British
FBS	Fetal Bovine Serum
FCS	Fetal Culf Serum
FISH	fluorescence in situ hybridization
FLT3	FMS-related tyrosine kinase 3
FRET	Fluorescence Resonance Energy Transfer
G-CSF	granulocyte - colony-stimulating factor 1
HSCT	haematopoietic stem cell transplantation
IgH	immunoglobulin heavy chain
IL	Interleukin
ITD	internal tandem duplication
JAK	Janus Kinase
JH	Janus Homology

MCSF	macrophage colony stimulating factor
MDR	multidrug resistance
MDS	myelodysplastic syndrome
MMM	myelofibrosis with myeloid metaplasia
MPD	myeloproliferative disease
MPO	myeloperoxidase
MRD	minimal residual disease
NHR	nervy homology regions
NK	normal karyotype
NPM	nucleophosmin
OS	overall survival
PB	peripheral blood
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PLT	platelets
PV	polycythemia vera
RAS	ras-sarcoma protooncogen
RFS	relapse-free survival
RHD	Runt homology domain
RQ-PCR	Real time Quantitative PCR
RT	reverse transcription
RTK	receptor tyrosine kinase
RT-PCR	Reverse-Transcriptase PCR
RUNX1	Runt-related transcription factor 1
STAT	signal transducer and activator of transcription
Taq	thermus aquaticus
TCR	T-cell receptor
TK	tyrosine kinase
Tm	melting temperature
WBC	white blood cells
WHO	World Health Organization
WT	Wilms tumor

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Ich erkläre hiermit an Eides statt,

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DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA WITH t(8;21) TRANSLOCATION

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