Clinical Relevance of Genetic Alterations in Acute Myeloid Leukemia in Children with Down syndrome

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KLINISCHE RELEVANTIE VAN GENETISCHE AFWIJKINGEN BIJ ACUTE MYELOÏDE LEUKEMIE BIJ KINDEREN MET DOWN SYNDROOM

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CHAPTER 1

GENERAL INTRODUCTION

HEMATOPOIESIS AND LEUKEMIA

Hematopoiesis is the formation of new blood cells, which is a tightly balanced and highly organized process of proliferation, differentiation, maturation and cell survival [1]. This process starts in the yolk sac during embryonic development. As the development of the embryo progresses, blood cell formation continues predominantly in the liver, and after birth the bone marrow takes over the process of hematopoiesis. All types of blood cells develop from a communal pool of self-renewing hematopoietic stem cells (HSC's). In a strictly regulated process, the multipotent HSC's can differentiate into either common myeloid or common lymphoid progenitor cells (Figure 1). Lymphoid progenitor cells will have the ability to differentiate and mature into either B-lymphocytes or T-lymphocytes, whereas the myeloid progenitor cells differentiate towards the other white blood cells (leukocytes) such as granulocytes (i.e. neutrophils, basophils and eosinophils), monocytes/macrophages and mast cells, but also to the red blood cells (erythrocytes) and the platelets (thrombocytes).

Leukemia (from the Greek 'leukos' means white and 'haima' means blood) is cancer of the blood cells characterized by uncontrolled proliferation of immature white blood

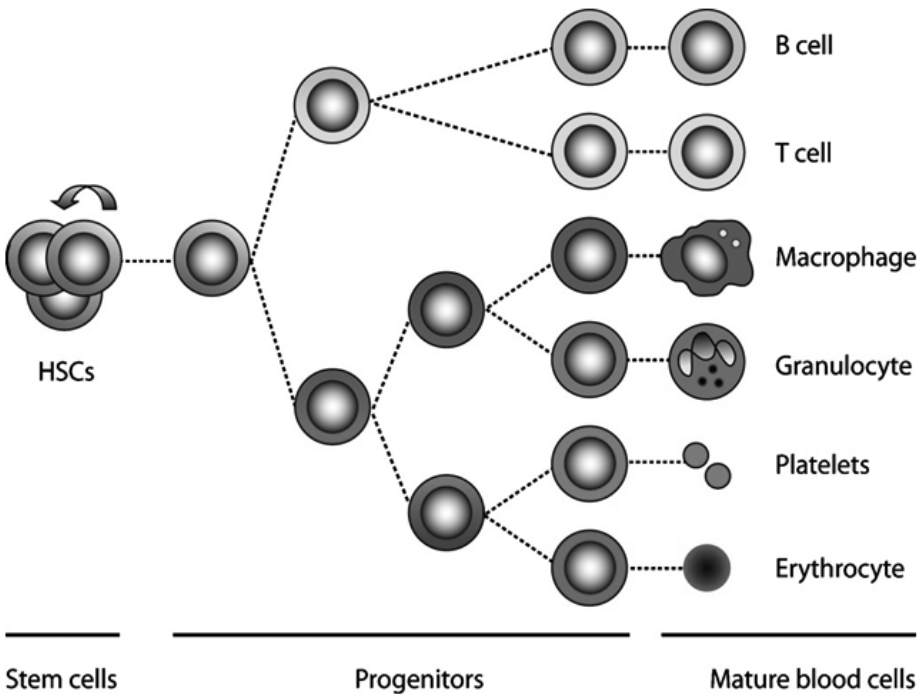

Figure 1. Schematic overview of hematopoiesis.
HSC= hematopoietic stem cell (see color figure on page 155)

cells (blasts) in the bone marrow. Consequently, normal hematopoiesis fails and patients suffer from the lack of normal blood cells [1]. Different types of leukemia are defined by the lineage of the progenitor cell that was subject to malignant transformation. Lymphoblastic leukemias are derived from the lymphoid lineage and myeloid leukemias originate from the myeloid progenitor cell. Leukemias can further be classified either in an acute or chronic type. Acute leukemias are rapidly progressing and characterized by the clonal expansion of hematopoietic progenitor cells caused by a maturation arrest combined with enhanced proliferation resulting in a fast increase of immature blood cells. In contrast, chronic leukemias result from hyperproliferation without a clear maturation arrest and usually develop more slowly. Following this, four major types of leukemia can be distinguished: acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL). Chronic leukemias are very rare in children. ALL comprises the largest part (75-80%), and AML accounts for 15-20% of pediatric leukemias [2].

DOWN SYNDROME

Down syndrome (DS) reflects one of the most common germline chromosomal abnormalities. It is caused by trisomy 21 (Hsa21), which is either the result of non-disjunction in the first or second meiotic division (>95% of the cases) or caused by a Robertsonian translocation (2-3% of all cases), i.e. a translocation resulting from the fusion of the whole long arms of 2 acrocentric chromosomes. The majority (~90%) of the non-disjunctions is of maternal origin. Only 5% of all DS individuals have mosaicism or Robertsonian translocations which is associated with none or less symptoms.

Clinically, DS is characterized by several dysmorphic features and delayed psychomotor development. Children with DS also have an increased risk of concomitant congenital heart defects and organic disorders such as gastrointestinal defects, celiac disease, impaired immunity with subsequent higher infection risk, and hypothyroidism. The life expectancy of children with DS is primarily dependent on the risk of mortality in the first year of life [3].

The incidence of trisomy 21 is influenced by maternal age and differs between populations (between 1 in 319 and 1 in 1000 live births) [4-6]. This incidence has decreased over the past several decades due to the increased prenatal screening and selective termination of DS pregnancies [3].

DOWN SYNDROME AND LEUKEMIA

Children with DS have an increased risk of developing leukemia, which was already recognized in the 1930s [7-10]. This increased risk of leukemia includes both the risk for acute lymphoblastic leukemia (ALL), which is approximately 20-fold higher, as well as acute myeloid leukemia (AML), which is 150-fold higher [11], as compared to non-DS children. The incidence of acute megakaryoblastic leukemia (AMKL) in particular is estimated to be 500-fold higher in children with DS compared to the general population [12].

This remarkable strong predisposition for DS children to develop acute leukemias is not completely understood. Several studies have examined potential leukemogenic environmental factors. It has been suggested that exposure to infections at early age may protect against the development of ALL in children in general [13, 14]. In DS individuals, however, no association was found between acute leukemia and common infections occurring at early age [15, 16] or maternal infections during pregnancy [15, 17]. Peri-conceptional vitamin use [18] and maternal infertility treatment [19] may potentially contribute to leukemogenesis in DS children, although these hypotheses need to be confirmed further in epidemiological studies.

Interestingly, DS is not a classic genomic instability syndrome with a general increased risk for developing cancer, as there is a reduced propensity for solid tumours [8]. In addition, the frequency of secondary malignancies after treatment for prior leukemia is decreased [11]. One or more of the trisomic genes on chromosome 21 might be responsible for this phenomenon. E.g. the *DSCR1* (Down's syndrome candidate region 1) gene, which encodes a protein that suppresses vascular endothelial growth factor (VEGF) mediated angiogenic signaling. As angiogenesis is essential for tumour growth, its suppression by overexpression of *DSCR1* [20] could impair tumour expansion, and thereby reduce the occurrence of solid tumors in individuals with Down's syndrome. An exception to the decreased risk of developing solid tumours are germ cell tumours, especially testicular tumours, but this may be due to the high incidence of undescended testes in boys with DS [21].

TRANSIENT MYELOPROLIFERATIVE DISORDER

Transient myeloproliferative disorder (TMD) or transient leukemia (TL) is a clonal disease that is characterized by immature megakaryoblasts in the fetal liver and peripheral blood [22, 23]. The relation between transient leukemia and DS has already been described in the first half of the previous century when it was reported as a congenital condition [24, 25]. Honda *et al.* was one of the first to describe that this leukemia had a transient character that could give rise to overt leukemia later in life [26]. The true frequency of

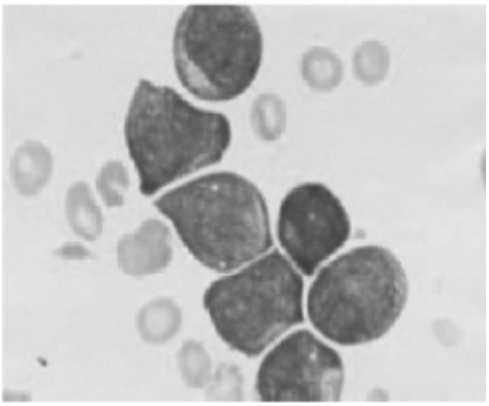

Figure 2.
Blasts in peripheral blood (see color figure on page 155)
(May-Grünwald-Giemsa staining)

TMD is not known because it is only studied in selected populations [27]. As the course of TMD is uncomplicated and asymptomatic in the vast majority of cases, a diagnosis of TMD is often missed prior to its spontaneous resolution. The frequency of TMD is probably lower than estimated in older series (who claimed 20%) which were based on hospitalized children with complications. We assume that it is less than 10% [28], which is supported by the finding of *GATA1* mutations (see below) in 590 Guthrie cards in 3.8% of newborn DS children [28]. However, mutations may have been missed due to subclonality, low numbers of cells or blasts (which hampers the detection), or extramedullary disease without circulating blasts. In addition, some DS foetuses may die in utero from causes including TMD [29]. Previous studies are not population-based and thus the true incidence of TMD within DS patients cannot be directly ascertained. A population-based prospective study is currently ongoing within the Dutch Childhood Oncology Group.

TMD develops antenatally, so its presence can be suggested by ultrasound findings of fetal hepatomegaly and/ or hydrops foetalis (and can also be detected by fetal blood sampling), but is usually diagnosed within a few days after birth [30-32]. Within in the liver, there is megakaryocytic infiltration and liver fibrosis, likely caused by excess cytokines secreted from the megakaryoblasts [33]. TMD exhibits megakaryoblastic morphology and the immunophenotype is indistinguishable from ML-DS, but the natural history of TMD is one of spontaneous resolution [34]. It is hypothesized from animal studies that this spontaneous resolution of the TMD-clone is concomitant with the switch of the hematopoietic microenvironment from liver to bone marrow [35].

Clinical presentation and treatment

There is a broad variety in the clinical presentation of TMD. Symptoms range from asymptomatic disease to massive organomegaly and fetal liver and/ or respiratory failure,

which may lead to death in approximately 10-20% of cases [36, 37]. The largest prospective study to date studied 146 cases with TMD (children with morphologic evidence of >5% blasts were included); the median age at diagnosis was 3 days (0-65), the median WBC was 40.3 x10^9/l, and 66% of all DS children showed spontaneous remission [36]. Most patients presented with hepatomegaly (60%), splenomegaly (42%), pleural/pericardial effusions (23%), or ascites (12%). Only 14 (10.2%) children did not have relevant clinical symptoms at diagnosis. A total of 7 children with TMD diagnosed after birth suffered from hydrops fetalis. Furthermore, the authors showed a correlation between high white blood cell (WBC) count, the presence of ascites, preterm delivery, bleeding diatheses, failure of spontaneous remission, and the occurrence of early death (9%). Treatment with low dose cytarabine (0.5-1.5 mg/kg for 3-12 days) was administered to 28 patients with high WBC count, thrombocytopenia, or liver dysfunction. Therapy (with low dose subcutaneous or intraveneous cytarabine) had a beneficial effect on the outcome of those children with risk factors for early death [36].

As TMD originates in the liver, peripheral blood blast percentages are usually higher than the bone marrow percentages. Therefore, bone marrow aspirates are not necessary and add little to peripheral blood analysis when the diagnosis is indicated by clinical findings and peripheral blood examination [34, 38]. The course of TMD is that it usually resolves spontaneously within the first 3 months of life. However, several studies revealed that the disease may not always have a benign clinical course, as early death is reported in 10% to 20% of affected children. It needs to be mentioned that most studies did not prospectively screen all infants with DS for TMD, but rather included only those who were diagnosed with TMD, and this contributes to the relatively high morbidity and mortality rate related to clinically evident TMD.

MYELOID LEUKEMIA OF DOWN SYNDROME

Clinical characteristics

Myeloid Leukemia of Down Syndrome (ML-DS) is a unique disease entity and its clinical and biological characteristics differ from AML in non-DS children, and is therefore recognized as a separate entity in the new WHO-classification [39, 40]. Approximately 70% of all ML-DS cases have an excess of megakaryoblasts as in acute megakaryocytic leukemia (AMKL, FAB M7). Furthermore, ML-DS is characterized by a low diagnostic white blood cell count, young age, typically between 1 and 4 years [41] and *GATA1*-mutations (see below) are pathognomonic [42, 43]. CNS involvement is found in 1-3% of all children with ML-DS [41, 44], which is less frequent compared to non-DS AML pediatric studies (2%-5%)[45, 46].

Hasle *et al.* [47] showed that only 2 out of the 12 DS children over 4 years of age with AML had a *GATA1*-mutation. AML cases in DS children lacking this mutation are therefore considered to suffer from 'sporadic AML' in DS instead of the unique ML-DS. Therefore, it is suggested that AML in DS children should be classified based on the *GATA1*-status as its unique molecular genetic basis. In addition, as mosaic DS children may lack the specific DS phenotype, *GATA1* mutated leukemia has to be excluded in all young children with AM(K)L (<5years old) to prevent overtreatment [48].

Treatment and outcome

Before the 1980's, children with ML-DS were often treated with palliative care only. Thereafter, stable remissions in ML-DS patients who were treated with chemotherapy were reported [49, 50]. However, when full dose chemotherapy was applied, many DS children developed severe side-effects, and the incidence of treatment-related mortality was increased compared to sporadic pediatric AML [51].

It is known that ML-DS blasts *in vitro* are relatively chemosensitive compared to their non-DS counterparts [52]. Specific mechanisms have been suggested to explain this sensitivity by linking it to a gene-dosage effect of chromosome 21 localized genes [53]. E.g. cytarabine sensitivity may be caused by increased levels of the cystathionine-B-synthetase gene, which is located on chromosome 21 [54, 55]. The presence of GATA1s has been shown also to result in higher cytarabine sensitivity [56]. However, as the increased chemosensitivity *in vitro* has been described for drugs that act in different ways, this suggests a more general mechanism of enhanced chemotherapy susceptibility, such as a propensity to undergo apoptosis [52]. Another hypothesis is that the presence of GATA1s leads to an altered response to chemotherapy exposure [57] as *GATA1* interacts and regulates the expression of different genes related to drug metabolism [58]. The increased chemosensitivity does not only apply to the leukemic cells but also to the healthy tissues and thereby contributes to increased treatment related morbidity and mortality [59]. Thus, several study groups established that, to reach good survival rates in children with DS, it is crucial that high intensity treatment (required for cure of non-DS AML) is avoided [50, 59, 60]. Therefore, specific treatment guidelines for ML-DS patients have been developed, with reduced treatment intensity regimens. Based on the promising results (3-years OS 90%) in the AML-BFM-98 study, a prospective European treatment protocol (ML-DS 2006) was opened with the aim to achieve at least 85% overall survival. In this protocol, ML-DS patients will receive the standard BFM chemotherapy regimen, with significant dose-reductions for cytarabine and anthracyclines. Children with ML-DS, suffering from relapse should be treated according to an individualized schedule, which takes the increased risk of toxicity and potential resistant disease into account. Stem cell transplantation should not be a standard practice, given the high risk of procedure related morbidity and mortality in DS children (ML-DS 2006 protocol). Apart from dose

reductions, the development and implementations of extensive supportive care has contributed to better survival of ML-DS patients.

GATA1

The *GATA1*-gene is a double zinc finger DNA-binding transcription factor that forms distinct activating or repressing complexes with partner proteins [61-63]. *GATA1* is specifically active in hematopoiesis and is essential for normal erythroid and megakaryocytic differentiation [64-66]. Constitutional mutations in *GATA1* have been described in disorders that cause variable degrees of anemia of thrombocytopenia, but these are not associated with the development of leukemia [67-69]. Since *GATA1* is located on the X-chromosome this follows an X-linked inheritance pattern.

The acquisition of *GATA1* mutations is an essential step in the leukemogenesis of TMD and ML-DS, as selective loss of megakaryocytic *GATA1* expression led to thrombocytopenia associated with deregulated megakaryocytic proliferation and maturation [70]. These unique, somatic mutations, initially described by Wechsler *et al*. [43], all leading to the N-terminus truncated protein GATA1short (GATA1s; 40kDa), that is translated from an alternative downstream initiation site (Figure 3).

It was shown that the short GATA1s isoform could not properly restrict megakaryocyte growth compared to full length GATA1 [71, 72] and results in excessive proliferation and, although to lesser extent, abnormal differentiation of an embryonic fetal megakaryocyte progenitor population [73]. As *GATA1* is encoded on the X-chromosome, only the mutant allele is expressed in both males and females (due to X-inactivation) [74]. The mutations occur mainly in the 5' region of the gene in exon 2, and to a lesser extent in exon 3. Most mutations are small insertions, duplications, deletions or point mutations, although rare cases with large deletions have been reported.

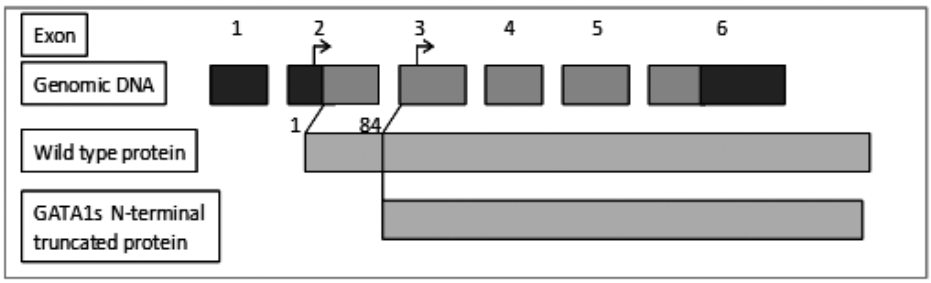

Figure 3.
Schematic overview of mutations in the *GATA1*-gene. (see color figure on page 156)

GATA1 and trisomy 21

GATA1 mutations occur both in TMD and in ML-DS [42, 75-81]. Paired sequencing data of 4 children from whom material was available for both TMD and ML-DS revealed the same *GATA1* mutation in the paired cases, suggesting clonal stability [36]. Trisomy 21 by itself affects fetal hematopoiesis, and the expansion of fetal liver progenitors creates a cellular substrate for *GATA1* mutations, which subsequently gives rise to TMD [82]. However, GATA1s is insufficient to induce leukemia in the absence of trisomy 21 in either humans or mice [73, 83].

GATA1 mutations are early events in the multi-step leukemogenesis (Figure 4) of TMD and ML-DS and arise prenatally based on knowledge from detection studies in fetal tissue as well as retrospective detection in Guthrie newborn screening cards from infants who developed ML-DS later in life [28, 80]. This multi-step hypothesis is supported further by a pair of identical twins diagnosed with TMD whom had identical *GATA1* mutations in their leukemic cells, suggesting an in utero origin of these mutations [79]. Successful detection of *GATA1* mutations using real-time quantative polymerase chain reaction has been demonstrated and may be used as a marker for minimal residual disease (MRD). However, this is complex as mutations are patient-specific and not disease-specific, so sensitivity needs to be tested for each patient specifically [22]. Although unique for every patient, some types of *GATA1*-mutations have been significantly associated with lower white blood cell counts and a higher rate of progression to ML-DS after TMD [84], however this was not confirmed by others [85].

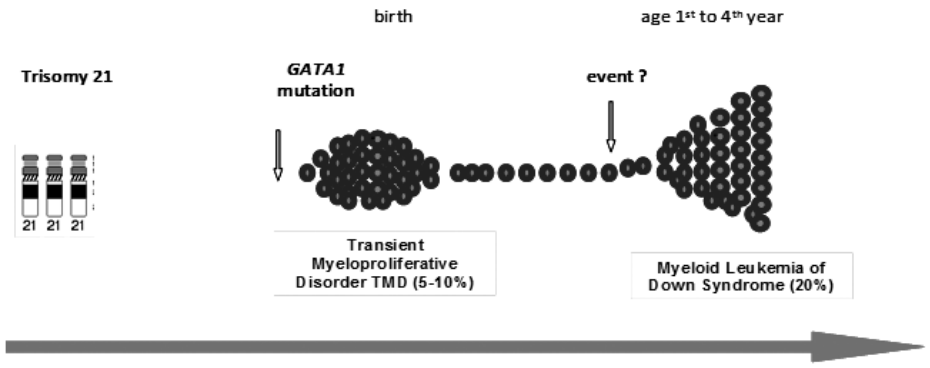

Figure 4.
Schematic overview of ML-DS leukemogenesis. (see color figure on page156)

Other genetic aberrations

Non-DS pediatric AML results from a multistep pathogenesis through the accumulation of different (epi-) genetic aberrations. In 2002, Kelly and Gilliland introduced the two-hit model in which they postulated that at least two cooperative types of events have to occur in a hematopoietic progenitor cell to result in acute leukemia [86]. Type-I aberrations lead to uncontrolled proliferation and/or survival, and are often activating mutations in signal transduction molecules. Type-II aberrations primarily impair normal differentiation, and typically results from aberrations of hematopoietic transcription factors such as fusion genes resulting from chromosomal rearrangements. Evidence for this type-I/-II model is supported by the fact that in AML usually one known type-I aberration and one known type-II aberration are present [87]. In addition, type-I and type-II aberrations are associated in a non-random fashion, suggesting cooperation between these specific aberrations [87]. Lastly, it has been shown in mice models that one aberration is not sufficient to induce leukemia, hence both types of aberrations are mandatory to develop an overt AML [88]. These aberrations can be identified by diverse molecular techniques, such as direct sequencing and fragment length analyses. At the time of start of the work described in this thesis the frequency of these mutations in ML-DS was not precisely known. Recently, mutations that affect the epigenetic landscape, i.e. DNA methylation status or histone code were found in non-DS AML. These aberrations might not so strictly be distinguished as currently being done in the classical type-I/-II model and thus might point towards other processes that may play a role in the pathobiology of AML.

GENOME-WIDE APPROACHES TO FURTHER UNRAVEL TMD AND ML-DS

Several high-resolution genome-wide array-based techniques have become available in the last decade to further study the genetic landscape of different types of leukemia. Small chromosomal copy number alterations such as amplifications and deletions below the detection limit of conventional karyotyping can now be made visible by high resolution array-based comparative genome hybridization (A-CGH) and single nucleotide polymorphism arrays (SNP-A) at a resolution up to 0.7 kilo base pairs (kb). During the work described in this thesis it became clear that copy number alterations appeared to be relatively frequent in ML-DS compared to TMD and pediatric non-DS AML. Microarray-based gene-expression profiling (GEP) is a technique to measure the expression levels of thousands of genes at once using a single micro-array chip. It proved to be a powerful tool for classification and prediction of tumor subtypes (class prediction), identification of novel subtypes (class discovery), and providing insight in the pathobiology of a disease and its subtypes (class comparison)[89, 90]. In pediatric and adult AML gene expression profiles could predict the subtypes of AML such as t(8;21),

inv(16), t(15;17) and *MLL*-rearrangements with a very high accuracy [91-95]. Other GEP applications include for example the study for its ability to predict prognosis and the correlation with drug resistance or sensitivity [93, 96-99].

The development of next-generation or high-throughput sequencing technologies in the past years have paved the way to discover the complete genetic landscape of diseases. The publication of the first two completely sequenced whole AML genomes in 2008 and 2009 was the start of the discovery of novel pathogenic mutations in AML[100, 101] and hence may serve as an example for the unraveling of ML-DS. As whole genome sequencing has become relatively affordable nowadays but still goes along with enormous datasets, restriction of sequencing to the complete coding region of the genome (whole exome sequencing) might be a alternative option [102]. In this thesis we applied A-CGH and GEP to detect new genetic factors contributing to TMD and ML-DS.

OUTLINE OF THIS THESIS

Trisomy 21 and *GATA1* mutations are likely early steps in the multi-step leukemogenesis of TMD and ML-DS. Approximately 20% of TMD-patients develop ML-DS later in life and as there is no difference between *GATA1*- mutations at TMD and ML-DS, hence additional events are needed for this progression. Therefore, the work in this thesis focuses on the contributing genetic abnormalities in TMD and ML-DS leukemogenesis. In addition, prognostic (cytogenetic) subgroups within ML-DS are established.

In **chapter 2** we studied copy number alterations (CNAs) by using A-CGH in ML-DS patients and compared the results with non-DS AML cases. We aimed to detect new abnormalities specifically for ML-DS. In addition, we studied whether the high frequency of CNAs (and thus genomic instability) was related to telomere length or mutations in *TERT* or *TERC*. Thereafter, we performed mutational screening of *Janus kinases 1-3* (*JAK)* in TMD and ML-DS. *JAK* belong to a family of intracellular non-receptor protein tyrosine kinases that transduce cytokine-mediated signals via the STAT family of transcription factors and plays an important role in regulating the processes of cell proliferation, differentiation and apoptosis in response to growth factors (**chapter 3**). We also included a series of DS ALL patients. Furthermore, we investigated the prognostic impact of the *JAK*-mutations in our cohort. In **chapter 4** the frequencies of the well-known non-DS AML type-I and type-II aberrations were studied. We aimed at describing their frequency in ML-DS. In the next chapter **(chapter 5)** we addressed the question if there are any (cytogenetic) prognostic subgroups within ML-DS. Most ML-DS cases are characterized by additional karyotypic changes besides the constitutional trisomy 21. We studied the cytogenetic aberrations of ML-DS patients in a large international collaborative study and related those to outcome, thereby focussing on the cumulative incidence of relapse.

All karyotypes were centrally reviewed. We aimed to identify new prognostic groups which may result in risk-group stratification and risk-group directed therapy for these patients in the future. Lastly, we investigated whether TMD and ML-DS have specific gene expression profiles and to detect differentially expressed genes that may play a role in the evolution from TMD into ML-DS. Subsequently, we focused on *MLLT3* (myeloid/lymphoid or mixed-lineage leukemia, translocated to, 3), as this gene cooperates with *GATA1* in regulating the cell fate of the erythrocytes and megakaryocytes, and studied the functional role of this gene *in vitro*. Furthermore, we determined whether gene expression signatures can independently predict ML-DS as a separate entity from other pediatric AML cases (**chapter 6**). **Chapter 7** comprises the general discussion of this thesis and provides future perspectives.

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CHAPTER 2

HIGH FREQUENCY OF COPY NUMBER ALTERATIONS IN MYELOID LEUKEMIA OF DOWN SYNDROME

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ABSTRACT

Myeloid Leukemia of Down syndrome (ML-DS) is a unique disease entity. Most cases are considered to be of megakaryoblastic origin, as in non-DS acute megakaryocytic leukemia (AMKL). ML-DS can be preceded by transient myeloproliferative disorder (TMD) in newborns. Both diseases are characterized by mutations in the *GATA1*-gene.

We studied copy number alterations (CNAs) in TMD (n=16) and ML-DS (n=22), with the aim to study whether progression from TMD to ML-DS is associated with gains or losses of chromosomal material and to detect new abnormalities contributing to pathogenesis. Reference groups of non-DS AMKL and other pediatric AML-subtypes were used for comparison.

The number of patients with CNAs significantly increased when TMD was compared to ML-DS (p=0.026), and there was a trend for more CNAs per patient, both suggestive of clonal evolution. ML-DS had significantly more CNAs than other pediatric AML-subgroups, suggesting that the DS leukemias are genetically more unstable.

Genomic instability can be the result of critically short telomeres. Telomere length in ML-DS was slightly lower but not significantly different from telomere length in TMD and did not correlate with the frequency of CNAs. Mutations in *TERT* were present in 3/22 ML-DS patients, and in none of the TMD patients; however, there was no correlation with short telomeres.

INTRODUCTION

Pediatric acute myeloid leukemia (AML) is a heterogeneous group of leukemias that result from clonal transformation of hematopoietic precursors through the acquisition of chromosomal rearrangements [1, 2]. The development of AML arise from at least two different classes of cooperating genetic aberrations. Type I mutations (eg *KIT*, *RAS* and *FLT3-mutations*) confer a proliferative and/or survival advantage to hematopoietic cells, and type II mutations (e.g. the fusion genes *AML1/ETO* and *CBF/MYH11*) result in impaired differentiation [3].

Children with Down syndrome (DS) have a 150-fold increased risk of developing AML [4]. Myeloid Leukemia of Down syndrome (ML-DS) is a unique disease entity [5]. Most ML-DS cases are acute megakaryocytic leukemia (AMKL or FAB M7), and are characterized by a low diagnostic white blood cell (WBC) count, young age, a high sensitivity to chemotherapy *in vivo* and *in vitro vitro* (8-9), and excellent clinical outcome with survival rates of >90% [6-10]. Based on these data, protocols have been developed with reduced treatment-intensity [11]. These protocols result in a decrease in treatment-related mortality without increasing the relapse rate; together contributing to the improved outcome of ML-DS patients [7, 9, 11, 12].

ML-DS can be preceded by transient myeloproliferative disease (TMD) or transient leukemia in newborns. This clonal disease is characterized by expansion of immature megakaryoblasts in the fetal liver and subsequently in the peripheral blood [13]. Although TMD spontaneously disappears in most cases, it is estimated that approximately 20% of children diagnosed with TMD will develop ML-DS [14, 15].

The genetic aberrations commonly found in pediatric AML do not occur in ML-DS [16, 17]. However, both TMD and ML-DS blasts are characterized by somatic mutations in the *GATA-1* gene. This gene, which is localized on the X-chromosome, encodes for a transcription factor which regulates the differentiation of megakaryocytes and erythrocytes. Mutations lead to a truncated protein (GATA1s) and are unique for every patient [18-20]. Other factors that play a role in the pathogenesis of TMD and/or ML-DS are several overexpressed megakaryopoiesis-related genes encoded on chromosome 21 that may cooperate with excessive insulin-like growth factor (IGF) signalling in disturbing normal hematopoiesis in DS [21-23]. In addition, miR125-b-2, also located on chromosome 21, has been described as a potential oncomiR in these diseases [24]. As 20% of TMD patients develop ML-DS and 80% do not, it is hypothesized that additional (genetic) events are needed in the progression of TMD to ML-DS. These events are currently still unknown.

We used genomic profiling with the aim to detect new abnormalities (gene deletions and /or amplifications) involved in the pathogenesis of ML-DS, and to study the difference between TMD and ML-DS. A reference group of non-DS children with AMKL and other AML subtypes, i.e. cytogenetically normal AML (CN-AML) and *MLL*-rearranged

AML, were included for comparison. Although we did not detect recurrent amplifications or deletions, TMD and ML-DS were characterized by a relatively high frequency of copy number alterations, suggestive of genomic instability.

Genomic instability can be caused by short and dysfunctional telomeres [25]. Telomeres cap the ends of chromosomes to protect them from damage, but shorten with each cell division. To prevent critical telomere loss, highly proliferative cells express telomerase (encoded by *TERT*), a reverse transcriptase that elongates telomeres by using the telomerase RNA component (*TERC*) as a template [26].

In AML, short telomeres in the leukemic cell population are associated with an abnormal karyotype [27, 28]. Furthermore, constitutional loss-of-function mutations in *TERT* are present in patients with AML, and are associated with cytogenetic abnormalities and short telomeres [29].

In healthy individuals with DS telomere biology seems to be aberrant. In peripheral blood lymphocytes, a more rapid decline in telomere length is seen as compared to healthy controls unaffected by DS [30]. The accelerated telomere shortening in DS is already present in fetal life [31]. Based on the known relationship between short telomeres and genomic instability, and the apparent aberrant telomere biology in healthy individuals with Down syndrome, we hypothesised that telomere length might be related to the increased genomic instability in leukemic cells of DS patients, and might explain progression from TMD to ML-DS. We assessed the incidence of *TERT* and *TERC* mutations in ML-DS and TMD patients, and correlated mutational status with telomere length. Furthermore, we compared telomere length between TMD and ML-DS patients, and correlated telomere length with the frequency of CNAs in both groups.

DESIGN AND METHODS

Patient samples

Viably frozen diagnostic bone marrow or peripheral blood samples taken at diagnosis from both TMD and ML-DS patients (unpaired samples) were provided by the Dutch Childhood Oncology Group (DCOG), the Berlin-Frankfurt-Munster AML Study Group (AML-BFM-SG) and the Nordic Society for Pediatric Hematology and Oncology (NOPHO).

The patient cohort consisted of 38 patients in total (22 ML-DS and 16 TMD samples); of 21 patients material was available for Array Comparative Genomic Hybridization (Array-CGH) analysis, and of 36 patients for analysis of *TERT/ TERC* mutations and telomere length. Twenty-one karyotypes were available and were all reviewed by B.H.B. The results were compared with non- DS pediatric AML subgroups: non-DS AMKL (n=10), CN-AML (n=41) and *MLL*-rearranged AML (n=66).

Leukemic cells were isolated and purified as previously described [32]. All resulting samples contained ≥ 80% leukemic cells, confirmed morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) – stained cytospin slides after thawing.

Cells were lysed in Trizol reagent (Gibco BRL/Life Technologies, Breda, The Netherlands) and stored at -80°C. Genomic DNA and total cellular RNA was isolated as previously described [32]. Additional phenolchloroform extraction was performed and the DNA was precipitated with isopropanol along with glycogen (Roche, Almere, The Netherlands).

Institutional ethical approval for these studies had been obtained from Erasmus MC-Sophia Children's Hospital according to local laws and regulations, as well as informed consent from all patients.

Genomic profiling

We used Array-CGH for genomic profiling, which allows the identification of chromosomal regions of gains and losses with oligonucleotide probes [33]. Array-CGH analysis was performed on 7 TMD and 14 ML-DS samples with the human genome CGH 105-K oligonucleotide array (Agilent Technologies, Palo Alto, CA, USA), which consists of 99.000+ oligonucleotide probes that span both coding and noncoding sequences with an 232 bp overall median probe spacing. The samples of the control groups, non-DS AMKL and the CN-AML were analyzed with the same platform. For the *MLL*-rearranged samples, the human genome CGH Microarray 44A was used (Agilent Technologies, Palo Alto, CA, USA), which consists of approximately 40.000 60-mer oligonucleotide probes that span both coding and noncoding sequences with an average spatial resolution of approximately 35 Kb. From each patient, 2 µg DNA was pooled with 2 µg reference DNA and array-CGH was performed as previously described [33]. Micro-array images were analyzed with Feature extraction software (version 9.5; Agilent Technologies) and data were imported into Array-CGH analytics software (version 5.0, Agilent Technologies).

Copy number alterations (amplifications and/ or deletions) were identified as a minimum of 3 (44K arrays) or 5 consecutive probes (105K arrays) deviating beyond the threshold of 0.8 for single copy loss and 1.8 for bi-allelic loss. Large deletions were defined as a loss ≥0.5 million bases, whereas focal deletions were defined as losses < than 0.5 million bases [34].

Mutational analysis

TERT and TERC

Polymerase-chain-reaction (PCR) amplifications of the *TERT* and *TERC* gene were performed as previously described [35, 36], in 22 ML-DS patients and 14 TMD patients, including all but three patients that were also included in Array-CGH analysis. PCR products were purified with the EdgeBio ExcelaPure 96-Well UF Plate. Direct sequencing was performed

with BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA). Specific primers for sequencing were designed and sequencing products were analyzed in an automated genetic-sequence analyzer (ABI Prism 3100, Applied Biosystems). Sequences were determined in one direction, and mutations were confirmed in 2 independent PCR amplification products.

Telomere length measurement

Telomere length was assessed in genomic DNA extracted from blast cells by quantitative polymerase chain reaction (PCR) as previously described [37]. Telomere length was expressed as a telomere to single gene copy number (T/S) ratio, normalized to the T/S ratio of a reference sample, which was also used for the standard curve. Results were only accepted if the T/S ratio of the reference sample fell within a 5% variation. Telomere lengths were measured in triplicate in leukemic cells of DS patients with TMD (n=14) and ML-DS (n=22).

Statistics

For statistical comparisons the Statistical Package for the Social Sciences (SPSS) Analysis system (v.15.0, SPSS Inc., Chicago, IL, USA) was used. To compare categorical variables we used X^2 analyses and the Fisher exact test for small patient numbers. The nonparametric Mann-Whitney U test was applied for continuous variables. *P*-values less than or equal to $p < 0.05$ were considered statistically significant (two-tailed testing). Correlation parameters were determined with the Spearman correlation coefficient (R).

RESULTS

Study population

The 16 TMD-patients had a median age of 3 days at diagnosis (range 1-46 days) with a median WBC of 100 $x10^9$/l (range 35- 410 $x10^9$/l) and 75% were male. The median age at diagnosis of the 23 ML-DS patients was 1.9 years (range 0.7-2.5 years) and the median WBC was 9 $x10^9$/l (range 3-168 $x10^9$/l). Forty-one percent of the patients were male. Patient characteristics are summarized in detail in Table 1. The control groups consisted of 7 non- DS AMKL patients, as well as 66 *MLL*-rearranged AML and 41 CN-AML patients.

TMD and ML-DS

Common regions of CNAs were found on chromosome 1q (which harbored 6.0% of all CNAs) and on chromosome 11 (7.1%) in the ML-DS patients. This mainly consisted of large (sub) chromosomal amplifications, rendering the detection of a specific abnormality rather difficult. Schematic overviews of the CNAs are depicted in figure 1A and 1B.

Table 1 Clinical and genetic characteristics of the ML-DS patients

Pat nr	Disease	Sex	Age	WBC	Karyotype	Amp	Del	Total CNAs	*TERT* mutation	T/S ratio
1	ML-DS	male	1.3	6	47,XY,t(4;15)(q?21;q?21),del(7)(q?31q?33),+21c/47,XY,+21c	na	na	na	wt	1.17
2	ML-DS	female	0.7	4	na	0	1	1	wt	1.25
3	ML-DS	male	0.9	4.7	47,XY,+21c	0	3	3	wt	na
4	ML-DS	male	2.0	26	na	1	1	2	wt	0.53
5	ML-DS	female	2.2	9	na	2	3	6	wt	0.61
6	ML-DS	male	1.0	4	47~49,XY,+?19,+21c,+21[cp7]/47,XY,+21c[5]	na	na	na	wt	1.34
7	ML-DS	female	2.0	168	na	9	0	9	wt	0.57
8	ML-DS	female	2.0	6	48,XX,der(9)t(1;9)(q23;p22),+11,?del(13)(q14q21),+21c[18]	3	3	6	wt	0.94
9	ML-DS	male	2.3	12	47,XY,+21c	1	3	4	wt	1.16
10	ML-DS	female	1.9	11	47,XX,del(7)(p11.1),+21c[3]/47,XX,+21c[4]	0	9	9	wt	1.80
11	ML-DS	male	2.5	6	48,XY,t(9;14)(p21;q24),+11,der(16)t(1;16)(q22;p13),+21c[21]/47,XY,+21c[14]	na	na	na	wt	0.74
12	ML-DS	female	0.9	19	na	4	1	5	wt	0.76
13	ML-DS	male	1.8	7	47,XY,+21c[13]	1	1	2	wt	0.73
14	ML-DS	male	1.4	6	48,X,ins(Y;5)(q11;?),der(3)t(3;6)(q2?8;?) or ins(3;6)(q2?8;?),-5,del(6)(q1?4q2?4),+21c,+21,+mar[24]	1	7	8	A1062T	0.80
15	ML-DS	female	2.3	49	47,XX,der(9)inv(9)(p24q2?1)del(9)(q2q3),+21c[14]	0	3	3	wt	0.47
16	ML-DS	female	1.9	40	47,XX,der(1)t(1;1)(p36;q21),t(5;6)(p15;p23),+21c[23]	3	3	6	wt	0.64
17	ML-DS	female	2.4	3	47,XX,r(7)(p22q22).ish r(7)(WCP7+,D7Z1+,D7S486-,164D18-,3K23-),+21c	0	3	3	wt	0.83
18	ML-DS	female	2.2	7	45-46,XX,der(1)t(1;6)(q31;q?),ins(4;1)(q12;q25q44),-6,-7,der(7)t(6;7)(p21;p22),der(7)t(7;8)(q2?2;q2?3),der(11)t(7;11)(p14;p15),+21c,der(22)t(1;22)(q25;p11)[cp13]/47,XX,+21c[28]	na	na	na	A1062T	1.27
19	ML-DS	female	1.2	53	47,XX,?del(3)(q?26),add(17)(q?21),add(20)(q11),+21c[10]	na	na	na	wt	0.62
20	ML-DS	female	1.4	10	46,XX,dic(15;21)(p11;p11),+21c[10]/46,idem,t(3;5)(q24-q25;p15)[4]/46,idem,t(3;5)(q24-q25;p15),add(10)(p31~32)[6]	na	na	na	T726M	0.65
21	ML-DS	female	0.9	3	47,XX,+21c[13]	na	na	na	wt	1.12
22	ML-DS	male	1.8	21	47,XY,del(13)(q13q21),+21c[3]/47,XY,+21c[3]	na	na	na	wt	0.51

ML-DS=myeloid leukemia of Down syndrome; WBC= white blood cell count (*10_9/L); wt= wildtype; na= not available
Age in years; amp= amplification(s); del= deletion(s) (as detected by Array-CGH)
Telomere length was expressed as a telomere single gene copy number (T/S) ratio, normalized to the T/S ratio of a reference sample

We detected no other repetitive abnormalities (CNAs) that were characteristic of either the TMD and/ or ML-DS samples. In addition, we found a derivative of chromosome 1 in one patient, and an extra copy of chromosome 11 in another patient of whom we did not have Array-CGH data available.

In order to study the difference between TMD and ML-DS, we looked into the frequency of CNAs. The percentage of ML-DS patients with CNAs was significantly higher than that of TMD patients (100% vs. 57% of patients with CNAs; *P*= 0.026; Table 2). Also, when calculating the median number of CNAs per patient, ML-DS-patients had a trend to have a higher number of CNAs than TMD patients (median 4.5 vs. 1.0; *P* =0.06) (Table 2).

When analyzed separately, both in TMD as well as in ML-DS, deletions were more common than amplifications, although this difference was not statistically significant (TMD-patients 57% vs. 29%; *P*= 0.43 and ML-DS patients 94% vs. 63%; *P*= 0.31). In TMD-patients 67% of the amplifications were chromosomal and 86% of the deletions were focal. In ML-DS, most of the amplifications (54%) and deletions (64%) were focal.

ML-DS and non-DS AMKL

Comparing CNAs in ML-DS with these in a reference group of non-DS AMKL samples (n=10) showed that all cases of ML-DS as well as all non-DS AMKL patients had at least one CNA (Table 2). The median number of CNAs was 4.5 (range 1-9) for ML-DS, and 6.5

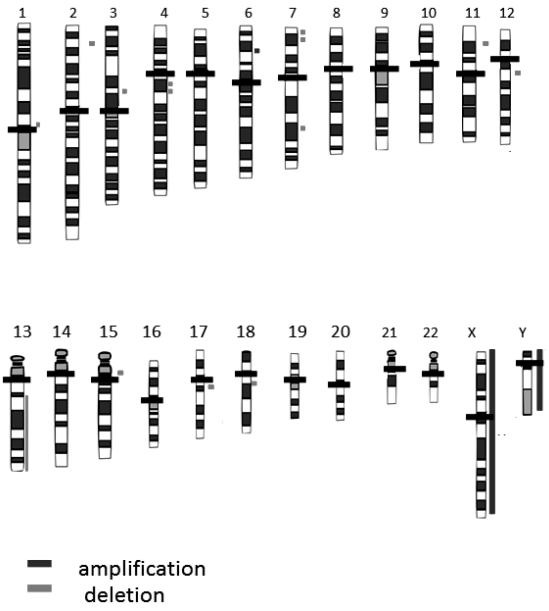

Figure 1A
Schematic overview of all CNAs taken together in TMD patients (n = 7). (see color figure on page 157)

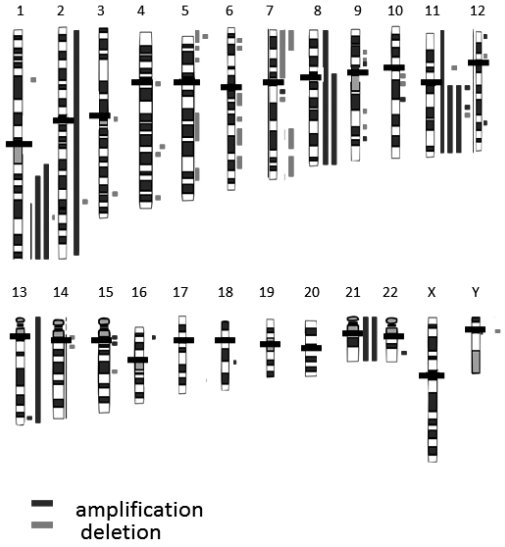

Figure 1B
Schematic overview of all CNAs taken together in ML-DS patients (n = 14). (see color figure on page 157)

Table 2
Copy number alterations (CNAs) in different subgroups of pediatric AML

Subgroup	%patients with CNAs	amplification	deletion	median number of CNAs per case
ML-DS n=14	100% *1, *2	64%	93%	4.5 *3, *4
non-DSAMKL n=10	100%	70%	80%	6.5
CN-AML n=41	37% *1	7%	34%	1.0 *3
MLL-rearrangedAML n=66	71% *2	38%	55%	1.9 *4

ML-DS = myeloid leukemia of Down syndrome;
non-DS AMKL = non-Down syndrome acute megakaryocytic leukemia;
CN-AML = cytogenetically normal AML
* indicates significant differences

*1 ML-DS vs. CN-AML: 100% vs. 37% ; P= 0.012
*2 ML-DS vs. *MLL*-rearranged AML: 100% vs. 71%; P= 0.033
*3 ML-DS vs. CN-AML: 4.5 vs. 0; P< 0.0001
*4 ML-DS vs. *MLL*-rearranged AML: 4.5 vs. 1.9; P< 0.0001

(range 1-18) for non- DS AMKL, which was not statistically significant (*P*= 0.58). In both groups deletions seemed to occur more common than amplifications (ML-DS 94% vs. 63%; *P*=0.31 and non-DS AMKL 80% vs. 70%, respectively, *P*=1.0; Table 2), but this was not significantly different.

ML-DS and other pediatric AML types

Seventy-one percent of the *MLL*-rearranged AML patients had at least one CNA (38% had ≥1 deletion(s) and 55% of the patients had ≥1 amplification(s)). The CN-AML group (n=41), had the lowest number of patients with CNAs of all the AML subgroups presented here: 37% in total; 34% had ≥1 deletion(s) and 7% ≥ 1 amplification(s).

The percentage of patients with CNAs was significantly higher in the ML-DS group than in the *MLL*-rearranged AML and CN-AML patients (100% vs. 71%; *P* = 0.033 and 100% vs. 37%; *P*= 0.012, respectively). The number of CNAs expressed per case was also significantly higher in ML-DS (median 4.5 (range 1-9.0)) patients compared to the *MLL*-rearranged subgroup (median 1.9 (range 0- 4.0); *P* < 0.001) and compared to the CN-AML patients (median 1.0 (range 0-9.0); *P*< 0.0001). Deletions seemed to be more common than amplifications (55% vs. 38%) in *MLL*-rearranged AML cases; *P*= 0.8 (Table 2) and in the CN-AML group (34% vs.7%; *P*= 1.0) but this was not significantly different.

TERT/ *TERC* mutations and telomere length

TERT mutations were detected in 3 out of 22 (14%) ML-DS patients and in none of the TMD patients (*P*= 0.16). Two patients carried an A1062T mutation, located in the C-terminal extension of *TERT*, and one patient carried a H412Y mutation, located in the N-terminal extension of *TERT*. Within ML-DS, all 3 *TERT*- mutants had a complex karyotype (≥ 3 aberrations), which showed a trend towards a higher frequency compared to the wildtype patients (3/3 versus 4/13, *P*=0.06). In only 1 of these 3 patients Array-CGH was performed. This patient, who carried an A1062T mutation in the leukemic cells, had a total of 8 amplifications and deletions. No *TERC* mutations were detected. Median telomere length in blasts for the patient with a mutated *TERT* allele, as reflected by T/S ratio, was 0.91; whereas the median for blast cells with wildtype *TERT* was 0.88 (*P*=0.6).

To assess whether the ML-DS group is characterized by a shorter telomere length than the TMD group, we measured telomere lengths in both groups. Median T/S ratio was 0.84 (range 0.5-1.23) in TMD- patients (n=14) and 0.76 (range 0.50-1.83) in ML-DS patients (n=22) (*P*= 0.9). No correlations were found between telomere length and the number of CNAs in TMD and ML-DS (correlation coefficient (R) 0.21; *P*= 0.78 and R 0.078; *P*=0.80, respectively). In addition, when CNAs were subdivided in amplifications and deletions, there were no correlations between telomere length and amplifications / deletions in TMD (NA and R 0.21; *P*= 0.78) and ML-DS (R -0.35; *P*= 0.25 and R 0.36; *P*= 0.2, respectively).

DISCUSSION

In our study, we detected a significantly higher number of CNAs in ML-DS patients compared to TMD, and a trend for a higher number of CNAs per patient. Although we did not study paired samples, this may indicate that clonal evolution contributes to leukemogenesis in DS children with TMD. At the moment, specific tumor suppressor or oncogenes involved in this progression have not been identified. However, the most common findings in our cohort of ML-DS patients were (sub-) chromosomal gains of chromosome 1q and chromosome 11. These chromosomes are both described to be aberrant in myeloid malignancies. Especially for chromosome 11, duplications are common (either the whole chromosome, or solely the p- or q-arm) in AML, particularly the 11q23 region [38]. Abnormalities on chromosome 1 have been found in different types of myeloid malignancies (e.g. unbalanced t(1;7) in AML) [39]. In addition, chromosome 1 and 11 aberrations have been identified previously by array-CGH in a smaller cohort (n=7) of ML-DS patients [40], but these aberrations were also found in single cases. A duplication of 1q and trisomy 11 are also frequently present abnormalities found in ML-DS patients [41].

Recently, by using single-nucleotide-polymorphism microarrays and candidate re-sequencing, it was shown that de novo pediatric AML is characterized by a low frequency of copy number alterarions (CNAs), with a mean of 2.38 somatic CNAs per case [42]. This was in contrast to pediatric acute lymphoblastic leukemia (ALL), with an average of 6 CNAs per case [43]. The only exception with a high number of CNAs in AML was AMKL (average of 9.33). In that study, only 9 AMKL cases were studied, of which only one patient had a *GATA1*-mutation, specific for ML-DS. The regions mostly involved in the CNAs in that study e.g. on chromosome 8, 9 and 16, differ with those commonly found in our patients. Chromosome 11 was also frequently involved, although these were deletions in contrast to amplifications in our study. These findings are in agreement with the fact that ML-DS is a separate disease entity.

We found high frequencies of CNAs (80%) are in our non-Down syndrome AMKL patients, which may suggest that genomic instability is a more general phenomenon of megakaryoblastic leukemias rather than of DS leukemia specifically. This is noteworthy, as these diseases highly differ in various other aspects. In non- DS AMKL outcome is poor, whereas outcome is very good in ML-DS patients [8, 11, 44]. In addition, ML-DS is characterized by mutations in *GATA1*, which do not occur in non- DS AMKL.

Furthermore, we showed that ML-DS cases have significantly more CNAs compared to other specific subtypes of non DS-AML types (i.e. CN-AML and *MLL*-rearranged AML). This may suggest that the leukemic cells of DS patients are more susceptible to genomic instability. However, DS itself is not a classic genomic instability syndrome. DS children do have an increased risk of developing leukemia, but the risk of developing solid tumours is lower, so the overall incidence of malignancies is not increased in DS [5].

Nonetheless, as healthy individuals with DS display accelerated telomere shortening [30], and as genomic instability can be the result of short and dysfunctional telomeres, we hypothesized that telomere length might be related to the increased genomic instability in leukemic cells of Down syndrome patients, and might explain the progression from TMD to ML-DS. In our study, telomere length, however, did not correlate with CNAs in either TMD or ML-DS. Furthermore, telomere length did not significantly differ between TMD and ML-DS. Three ML-DS patients carried *TERT* variants (A1062T and H412Y) that have been shown to decrease telomerase function, and to be associated with decreased telomere length [29, 36], but telomere length in mutant blast cells was not shorter than the average in wildtype *TERT* blast cells. Unfortunately, due to lack of germline material we were not able to assess the origin of these mutations. Hence, short telomeres do not seem to explain the differences in genomic instability (as reflected by CNAs) between ML-DS and TMD. Interestingly, we did see that all *TERT* mutants had a complex karyotype, indicative of genetic instability, whereas this was only 31% in wildtype patients.

In conclusion, within the Down syndrome patients, there was a significantly higher number of patients with CNAs in ML-DS compared to TMD, and a trend for more CNAs per patient. We showed that ML-DS had significantly more CNAs than other pediatric AML-subgroups, suggesting that the Down syndrome related leukemias are genetically more unstable. However, we could not prove our hypothesis that short telomeres underlie this genetic instability.

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CHAPTER 3

FREQUENCY AND PROGNOSTIC IMPLICATIONS OF *JAK 1-3* ABERRATIONS IN DOWN SYNDROME ACUTE LYMPHOBLASTIC AND MYELOID LEUKEMIA

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ABSTRACT

Children with Down Syndrome (DS) have an increased risk of developing acute lymphoblastic leukemia (DS ALL) and acute myeloid leukemia (ML-DS). ML-DS is often preceded by transient myeloproliferative disorder (TMD) in newborns, which in most cases resolves spontaneously. As most studies focus only on known mutations, the true frequency of *JAK* mutations in acute leukemias in Down syndrome may be underestimated. We performed mutational analysis of the whole kinase and pseudokinase domains of *JAK 1-3* by direct sequencing of 6 TMD, 14 ML-DS and 35 DS ALL samples and related this to outcome. *JAK1* mutations were found in 1 ML-DS patient and 1 DS ALL patient. One TMD patient and 1 ML-DS patient harbored a *JAK3* mutation. Six DS ALL patients had a *JAK2* mutation and their 10-year event free survival (EFS) was 100% vs. 75 +/-9 % in wildtype patients (p=0.27); the 10-year overall survival (OS) was 100% vs. 86 +/-7% (*p*=0.3) and the cumulative incidence of relapse (CIR) was 0% vs. 21 +/-9% (*p*= 0.32). Moreover, a large meta-analysis did not show any differences in survival of *JAK2* mutants compared to wildtype patients. In summary, *JAK* mutations are rare in DS-leukemias, except for *JAK2* mutations in DS ALL, which have no prognostic value.

INTRODUCTION

Children with Down Syndrome (DS) have an increased risk of developing leukemia, including both acute myeloid (ML-DS) and acute lymphoblastic leukemia (DS ALL) [1]. These leukemias differ in clinical characteristics and biology from leukemias in non-DS children. ML-DS is characterized by a low diagnostic white blood cell count (WBC), young age, FAB M7 morphology, excellent clinical outcome with survival rates of >90%, and a high sensitivity to chemotherapy in vivo and in-vitro [2, 3]. Reduced intensity treatment in ML-DS does not lead to an increase in relapse rates, however, due to a decrease in treatment related mortality, it does result in improved overall survival [4, 5]. ML-DS is often preceded by transient myeloproliferative disorder in newborns (TMD), which in most cases resolves spontaneously. Approximately 20% of TMD-patients subsequently develop ML-DS [6, 7]. Both the TMD and ML-DS blasts are characterized by mutations in the *GATA-1* gene, a hematopoietic transcription factor, which result in a truncated protein GATA1s [8]. Because these mutations occur both in TMD and ML-DS, additional genetic abnormalities are needed in the progression from TMD to ML-DS.

The prognosis of DS ALL patients is at best similar and often inferior to that of non-DS ALL patients [9, 10]. This is in agreement with findings from cellular cytotoxicity assays that showed that DS ALL cells do not have increased sensitivity to chemotherapy in vitro [3]. Consequently, in DS ALL dose-reduction should only be considered in case of unacceptable toxicity arising during treatment. Favorable prognostic factors in non-DS ALL such as high hyperdiploidy and *ETV6-RUNX1* gene-rearrangements are less frequently found in DS ALL, as well as unfavorable factors such as Philadelphia-chromosome or *MLL*-rearrangements [11, 12].

Janus kinases (*JAK)* belong to a family of intracellular non-receptor protein tyrosine kinases that transduce cytokine-mediated signals via the STAT family of transcription factors. *JAK* plays an important role in regulating the processes of cell proliferation, differentiation and apoptosis in response to growth factors. The *JAK2* V617F mutation is well-known in myeloproliferative neoplasms (MPN), and result in the impaired ability of the pseudokinase domain to negatively regulate the kinase domain [13]. The expression of *JAK2* V617F in mouse models leads to the development of a disease with a similar phenotype to polycythemia vera, with eventual progression to myelofibrosis, underscoring the role of this mutation in the pathogenesis of MPN [14, 15]. We and others have previously described activating mutations in *JAK2* and *JAK3* in TMD and ML-DS [16, 17]. Mutations within the pseudokinase domain of *JAK2* in DS ALL patients have also recently been reported. These activating *JAK2* R683 mutations occur at a different site than the V617F mutation, but both these mutations are localized in the pseudokinase domain and both have the same functional consequence, i.e. constitutive kinase activity [18-20].

As most studies only focus on known mutations in *JAK* [16, 17, 19-22], and hence may underestimate mutational frequency, we performed mutational analysis of the whole kinase and pseudokinase domains of *JAK 1-3* by direct sequencing. Moreover, we analyzed the prognostic significance of *JAK2* mutations in DS ALL in our cohort. As all cohorts of the prognostic impact of *JAK2* mutations in DS ALL are small, we collected all clinical data from these series [18, 19], and performed a meta-analysis, assuming that since most *JAK* mutations have been described to be activating mutations, they will result in increased proliferation and hence in increased relapse rates.

DESIGN AND METHODS

Patient samples

We screened 6 TMD, 15 ML-DS (TMD and ML-DS samples were unpaired) and 35 DS ALL samples taken at initial diagnosis. TMD and ML-DS patients were diagnosed between 1994 and 2007. DS ALL patients were diagnosed between 1992 and 2008 and were treated according to subsequent DCOG treatment protocols ALL 8, 9 and 10. Details of these treatment protocols have been reported elsewhere, except for protocol ALL10, which is currently ongoing [23, 24]. Clinical and cell-biological data, including cytogenetic data, were available for all mutated cases. Samples were provided by the Dutch Childhood Oncology Group, the AML-'Berlin-Frankfurt-Munster' Study Group, and the Nordic Society for Pediatric Hematology and Oncology. All study groups performed central review of the diagnosis, classification and clinical follow-up of the patients. All investigations had been approved by the Institutional Review Board, and informed consent was obtained according to local law and regulations.

Low-density mononuclear cell populations of bone marrow or peripheral blood were isolated after density gradient centrifugation of the sample using Ficoll Isopaque. All resulting samples contained 80% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)–stained cytospins. Genomic DNA was extracted using standard methods.

Mutation analysis

For mutational analyses, all exons encoding the kinase and pseudokinase domains of *JAK1* (exon 12-25), *JAK2* (exon 12-25) and *JAK3* (exon 12-24) were PCR amplified. All ML-DS and TMD samples were screened for *GATA-1* mutations in exon 2 and 3. Purified PCR products of *JAK 1-3* and *GATA-1* were bi-directionally sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems Inc., Foster City, CA, USA). The sequence data were assembled and analyzed for mutations using CLC Workbench version 3.5.1 (CLC Bio, Aarhus, Denmark).

Statistics

For statistical comparisons the Statistical Package for the Social Sciences (SPSS) Analysis system (v.15.0, SPSS Inc., Chicago, IL, USA) was used. *P*-values less than or equal to 0.05 were considered statistically significant (two-tailed testing).

To evaluate outcome, the following parameters were used: complete remission rate (CR), event-free survival (EFS), overall survival (OS) and cumulative incidence of relapse (CIR). CR was defined as less than 5% blasts in the bone marrow, with regeneration of tri-lineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere. Patients who did not achieve CR were considered as treatment failure on day 0. OS was measured from the date of diagnosis to the date of last follow-up or date of death from any cause. EFS was calculated from the date of diagnosis to the date of last follow-up or to the first event, including relapse, death in CR, and failure to achieve CR. The Kaplan-Meier method was used to estimate the 10 year probabilities of OS (OS), and EFS (EFS), and survival estimates were compared using the log-rank test. CIR (with treatment related death as competing event) was constructed by the method of Kalbfleisch and Prentice and compared by the Gray test. Statistical analysis was only performed when at least 5 patients were available per subgroup.

For the meta-analysis we collected the original outcome data from all participating studygroups and evaluated OS and EFS.

RESULTS

Patient characteristics

All 35 DS ALL patients were classified as B-cell precursor ALL and enrolled in consecutive DCOG treatment protocols (DCOG ALL 8, 9 and 10). The median age of DS ALL patients was 4.5 years (range 2.0-17.1 years), the median WBC was $8.7x10^9$/l (range $1.2\text{-}390x10^9$/l), and 51% of the patients were male. The median age of the ML-DS patients was 2.0 years (range 0.7-2.4 years), the median WBC was $8,0\ x10^9$/l (range $2.6\text{-}168\ x10^9$/l) and 36% were male. The TMD patients had a median age of 1.5 days (range 1-6 days) with a median WBC of $172.8\ x10^9$/l (range $35\text{-}410\ x10^9$/l) and 67% were male.

The median follow up time for survivors with DS ALL was 5.2 years (range 1.1 – 15.4 years) and for survivors with ML-DS 1.9 years (range 0-16.4 years). The patient characteristics of the TMD, ML-DS and DS ALL patients are described in detail in Supplementary Table S1 and Table S2.

Mutation analysis

GATA1 mutations were identified in all ML-DS and TMD cases. Mutations in *JAK1* were found in 1 (7%) ML-DS patient (D625R), and in 1 (3%) DS ALL patient (V651M). These

were both missense mutations leading to substitution of a single amino acid which are predicted to result in an altered protein. No events occurred in either of the patients with a follow-up of 2.4 and 3.1 years, respectively. See Figure 1 for the localization of the mutations in *JAK 1-3*.

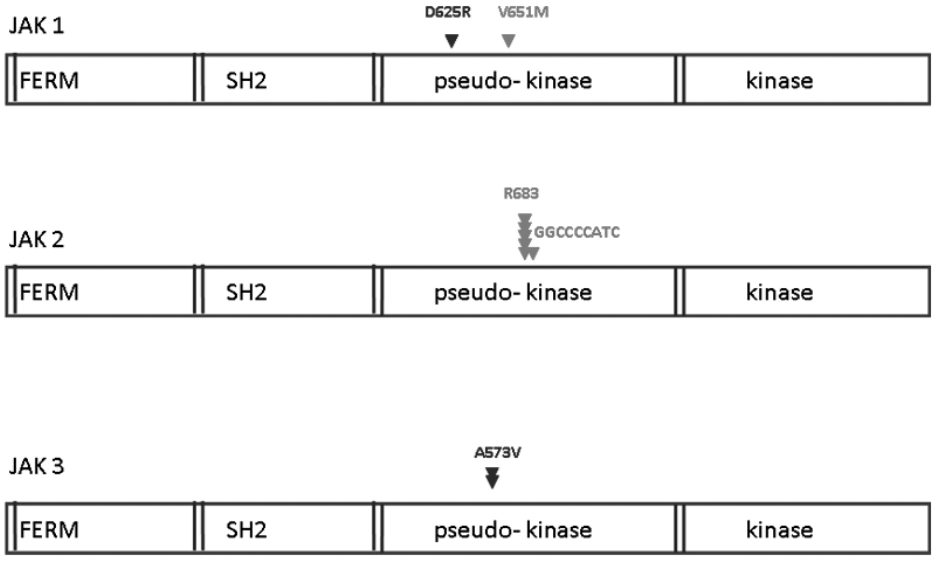

Figure 1
Localization of mutations in JAK 1-3. Schematic overview of the structure of *JAK 1-3* with the kinase, pseudokinase and FERM (4.1-ezrin-radixin-moesin) domain in which the location of the mutations we identified is indicated. Mutations in DS ALL are depicted in red, mutations in ML-DS and TMD in blue. (see color figure on page 158)

JAK2 mutations were not identified in any of the TMD and ML-DS patients. However, *JAK2* R683 activating mutations were found in 6/35 (17%) of the DS ALL patients. In 5 patients a substitution of nucleotides A à G was found which resulted in a substitution of arginine with glycine and 1 patient had an insertion of 9 base pairs (GGCCCCATC) immediately upstream of R683 (Supplementary Table S2). In DS ALL, cases with *JAK2* mutations were significantly younger than the wildtype patients, with a median age of 3.2 year vs. 4.9 years (p= 0.044). There were no significant differences in other characteristics such as WBC, sex, and cytogenetics between the *JAK2* mutated and the wildtype DS ALL patients.

One TMD-patient (17%) and 1 ML-DS patient (7%) harbored the *JAK3* A573V-mutation. Both patients are in continuous complete remission (CCR) with a follow up of 1.4 and 1.9 years, respectively.

Survival analysis

None of the 6 *JAK2* mutated DS ALL patients experienced an event, whereas 5 of the 29 patients with wildtype *JAK2* relapsed, and one died of treatment-related toxicity. Using Kaplan-Meier analysis (see graphs in Figure 2), no significant differences in clinical outcome were detected: the 10-year EFS for *JAK2* mutated versus *JAK2* wildtype patients was 100% vs. 75 +/- 9%; (p=0.27), the 10 year OS 100% vs. 86 +/- 7% (p=0.3). The cumulative incidence of relapse at 10 years after diagnosis in *JAK2* wildtype patients was 21 +/- 9% versus 0% in the *JAK2* mutated group; (p(Gray)= 0.32).

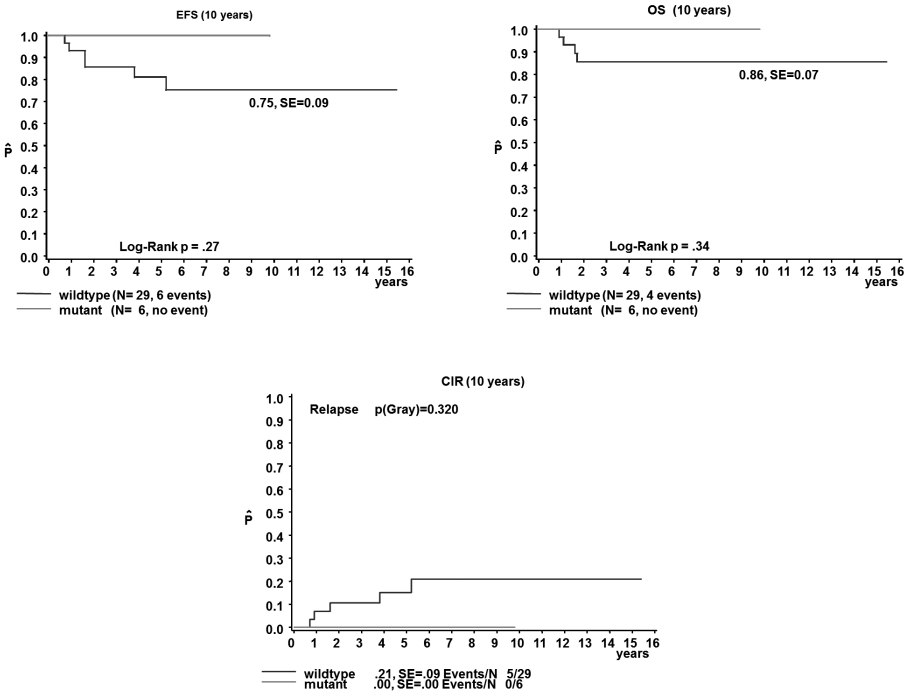

Figure 2

The 10-year survival parameters of *JAK2* R683 mutations in DS ALL patients. (see color figure on page 159)

(a) EFS (100% vs. 75±9%; P=0.27)

(b) OS (100% vs. 86±7%; P=0.30)

(c) CIR (0% vs. 21±9%; P=0.32)

Meta-analysis

To assess the impact of JAK2 mutations on the survival in a larger cohort of patients, we performed a meta-analysis of the data of 3 studies taken together pooling our data with those of Bercovich et al (17), and Gaikwad et al (18). This analysis did not show a statistical significant difference for the *JAK2* wildtype versus the *JAK2* mutated patients.

Six year EFS was 71 +/- 5% vs. 74 +/- 10%; *P*= 0.63 and OS was 76.0 +/- 4% vs. 89 +/- 6%; *P*= 0.30 (Figure 3A and 3B).

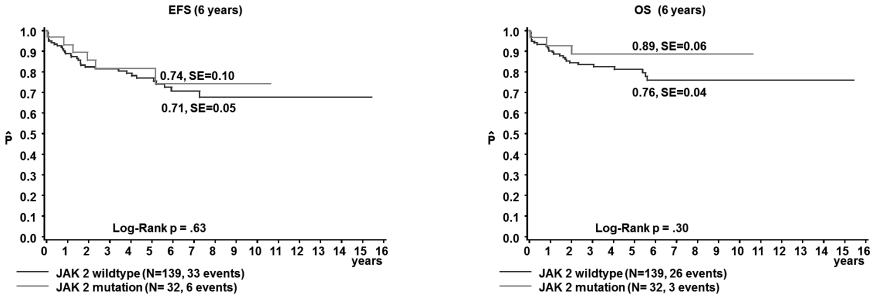

Figure 3

The 6-year survival parameters of *JAK2* mutant and wild type patients; meta-analysis of three datasets. DS ALL patients were diagnosed between 1992 and 2008. (see color figure on page 159)

(a) EFS 74±10% vs. 71±5%; *P*=0.63

(b) OS 89±6% vs. 76±4%; *P*=0.30

DISCUSSION

Our study focused on the frequency of mutations in all exons encoding the kinase and pseudokinase domains of *JAK 1-3* in DS leukemia patients. We did not detect a higher mutational frequency than previously described [16-22] suggesting that there are clear mutational hotspots which can be screened for in clinical studies. However, given the number of patients we have screened we may still have missed rare mutations that occur in a relatively low frequency in any of these leukemias.

Both *JAK1* mutations (one in ML-DS and one in DS ALL) which we found were localized in the same region of the pseudokinase domain, but not identical to the activating mutations in *JAK1* described in non DS ALL patients [25]. *JAK1* mutations in non-DS cases are mainly found in T-cell ALL (27.3 %) [26], and in low frequencies in adult ALL T-ALL and B cell precursor ALL (3.4%) [27] and AML (2.1%) [28]. These mutations are very heterogeneous in the sense that they are dispersed over several *JAK1* domains, and differ in their ability to transform hematopoietic cells and to activate downstream signaling pathways such as the STAT, PI3K and MAPK cascades [26-28]. T-cell ALL is exceedingly rare in DS patients, with frequencies varying between 0% and 7.8 % in several larger series [9, 29, 30], versus approximately 15% in non-DS ALL. No patients with DS and T-cell ALL were included in this study.

Recently, Bercovich and others reported activating *JAK2* R683 mutations in 18% of DS ALL patients [18-20]. This mutation was thought to be unique for DS ALL, in a similar fashion as *GATA-1* mutations are uniquely found in

ML-DS. However, the same *JAK2* mutations were also reported in non DS B-cell precursor ALL patients with a high risk for relapse, which implies that this mutation is not specific for DS ALL [25]. In our series, the typical *JAK2* R683 mutations were found in 17% of the DS ALL patients, which is in line with the frequency described by others [18-20]. One patient had an insertion of 9 base pairs immediately upstream of R683. This specific mutation has not been described before, but two different insertions of multiple base pairs at this position have been reported, and were considered to have an effect analogous to R683 mutations because of their location [18].

In contrast to the non DS B-cell precursor high-risk ALL patients with *JAK2* mutations, who were found to have a high relapse rate [25], none of the *JAK2* mutated DS ALL patients in our cohort experienced an event. The observed better outcome for *JAK2* mutated patients was, however, not statistically significant. So far, only two other studies report on the clinical relevance of *JAK2* mutations in DS-ALL and both studies are in agreement with our results reported here. Bercovich et al. reported a 5-year EFS of 73% in *JAK2* wildtype patients (n=62) versus 78% in *JAK2* mutated patients (n= 16), which was also not statistically significant [18]. In addition, Gaikwad et al. described a 5-year EFS in *JAK2* wildtype patients (n=43) of 76.3 % versus 87.5% in *JAK2* mutated patients (n=10), again a statistically non-significant difference [19]. A combined analysis of our data plus the 2 studies just mentioned above, showed no statistically significant difference in survival between *JAK2* mutated and *JAK2* wildtype patients. Since this meta-analysis includes 32 mutated cases and 139 wildtype cases, it provides substantially greater certainty that there is no survival advantage for DS ALL patients with a *JAK2* mutation.

JAK mutations play a role in activation of the JAK-STAT pathway, resulting in a proliferation advantage for leukemic cells [18], which led to our initial hypothesis that they would be associated with poor clinical outcome. One possible explanation for the observed good clinical outcome may be that this increased proliferation could contribute to enhanced sensitivity to chemotherapy. However, this is contradictory with the outcome of *JAK2* mutants in high-risk ALL, in which patients with a *JAK2* mutation have a high risk for relapse [25]. This may be due to currently unknown differences in step-wise leukemogenesis or cooperating genetic events between DS and non-DS *JAK2* mutated ALL.

Both one TMD patient and one ML-DS patient harbored a *JAK3* A573V-mutation. This activating mutation has previously been described in ML-DS patients and the megakaryoblastic cell line CMY [21]. It has been suggested that *JAK3* mutations may be associated with a more aggressive form of ML-DS [16]. However, our 2 patients with a *JAK3* mutation are in continuous complete remission (CCR) with a follow up of 1.4 and 1.9 years, respectively.

Of interest, *JAK* mutated cases may be sensitive to JAK-inhibitors. This might be of benefit, since it is well-known that DS patients have an increased risk for chemotherapy

related morbidity and mortality. In MPN, several JAK-inhibitors are already used [31]. Remarkably, patients with and without the *JAK2* V617F mutation may benefit to the same extent which is due to the fact that the current inhibitors do not differentially inhibit mutated and wildtype *JAK2*, because of the location of the mutation outside the ATP-binding pocket of the enzyme [18]. Treatment results in a decrease in organomegaly in responding patients [31, 32]. The major side-effect of JAK2 inhibitors is myelosuppression, which is due to suppression of wildtype *JAK2* that is required for normal hematopoiesis [13], and which may render it difficult to combine JAK inhibitors with chemotherapy. Given that *JAK2* mutated DS ALL cases do not have worse outcome it is difficult to predict whether the cells are addicted to the constitutive activity of *JAK2*, and hence whether inhibiting this will provide a benefit to these patients. We therefore feel that studies with *JAK2* inhibitors in DS ALL patients require further pre-clinical evidence of potential benefit before implementation.

Unfortunately, due to limited availability of our samples, we were not able to show activation of the JAK-STAT pathway at the protein level in the Down syndrome patients, and hence we may have missed patients that have JAK-STAT activation due to other mechanisms than mutations in JAK. Hence, further research is needed to identify potential JAK-STAT activation and its causes in patients without JAK-mutations. For instance, it is known that *MPL* (myeloproliferative leukemia virus oncogene) mutations may also activate the JAK-STAT pathway [13]. In an earlier study in 8 TMD and ML-DS patients these *MPL* mutations were not found [22].

In conclusion, *JAK1* and *JAK3* mutations are rare in DS-leukemias (although there seem to be mutational hotspots) whereas *JAK2* mutations occur relatively frequently in approximately 17% DS ALL cases. Of interest, none of the DS ALL cases with a *JAK2* in our cohort mutation relapsed so far, and a meta-analysis confirmed the lack of prognostic significance for *JAK2* mutated DS ALL patients.

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CHAPTER 4

LOW FREQUENCY OF TYPE-I AND TYPE-II ABERRATIONS IN MYELOID LEUKEMIA OF DOWN SYNDROME: UNDERSCORING THE UNIQUE ENTITY OF THIS DISEASE

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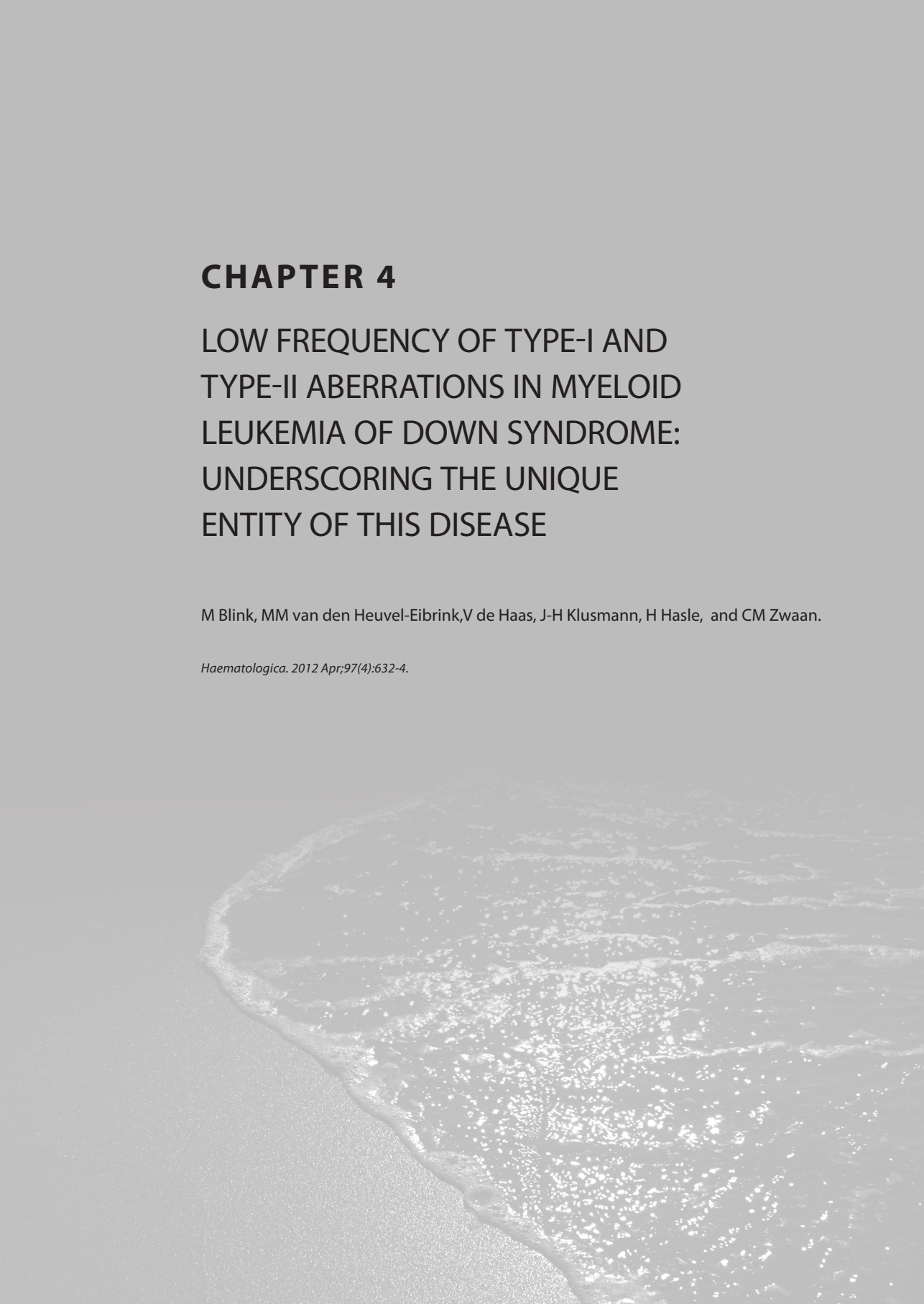

ABSTRACT

Type-I and type-II aberrations, involved in hyperproliferation and maturation arrest, are required for the development of acute myeloid leukemia (AML). These aberrations have been described widely in non-Down Syndrome pediatric AML patients, but have not systematically been investigated in Myeloid Leukemia of Down Syndrome (ML-DS). Therefore, we screened 34 *de novo* ML-DS patients for recurrent gene aberrations in the *NPM1*, *CEPBPA*, *FLT3, KIT*, *PTPN11*, *N-RAS*, *K-RAS*, *MLL*, *WT1*, *DNMT3A*, *IDH1*, and *IDH2* genes. Except for mutations in the RAS-pathway (*N-RAS* (3%) and *K-RAS* (7%)) and SNPs in the *WT1* and *IDH1* genes, we did not detect any aberration. This study underscores the unique signature of ML-DS and further research is needed to unravel other molecular aberrations involved in ML-DS leukemogenesis.

INTRODUCTION

Over the past decades the unraveling of the heterogeneous nature of pediatric acute myeloid leukemia (AML) has been successfully initiated. Recently, the currently known non-random collaboration of several genetic events, that induce respectively hyperproliferation (type I aberrations) and maturation arrest (type II aberrations) [1], required for the development of pediatric AML, has been extensively discussed in this journal [2]. However, the role of these type I/II- aberrations in Down Syndrome (DS) children that develop AML (ML-DS) has not yet been systematically investigated.

Non-DS AML accounts for 15-20% of pediatric leukemias [3]. This heterogeneous disease is reflected by differences in morphology, immunophenotype, as well as cytogenetic and molecular aberrations [4]. The so-called type-I aberrations are characterized as mutations in hotspots of specific genes involved in signal transduction pathways (*FLT3*, *KIT*, *N-RAS*, *K-RAS* and *PTPN11*), which lead to uncontrolled proliferation and/or survival of the leukemic cells. Type-II aberrations consist of chromosomal rearrangements, frequently involving transcription factors, which result in impaired differentiation of the leukemic cells (including *PML-RAR*A, *AML1-ETO, CBFB-MYH11,* and 11q23/*MLL*-rearrangements). These recurrent (cyto)genetic aberrations are also important prognostic factors in pediatric AML and are used for risk group stratification [5]. The non-random collaborative type I/ type II leukemogenic hypothesis was strengthened by observations from mouse models that one aberration is not sufficient to induce leukemia, but that cooperative events are needed to develop overt leukemia [6].

In the last decade, many novel genetic and molecular abnormalities in non-DS AML were discovered, including cryptic fusion genes, single-gene mutations, polymorphisms and overexpression of oncogenes. For instance, cryptic *NUP98/NSD1* translocations have recently been described as a frequent event in non-DS cytogenetically normal (CN) AML (16%), and were recognized as an independent predictor for dismal outcome [7]. Moreover, single gene mutations occurring in the *NPM1*, *CEBPA*, *WT1* and *MLL*-gene (MLL-PTD) which were mainly found in patients with CN-AML [8-11]. Clinically, *NPM1* and *CEBPA* double mutations were independent factors for favorable event-free survival (EFS), whereas *WT1* mutations combined with *FLT3*-ITD showed the worst outcome [2]. The recently discovered mutations in the metabolites *IDH1*/ *IDH2* and the DNA methyltransferase (*DNMT3A)* gene are rare in pediatric AML [12, 13]. To our knowledge, also no systematic study of these molecular aberrations has been performed in ML-DS patients so far.

Children with Down Syndrome (DS) have an increased risk of developing acute myeloid leukemia (ML-DS) [14]. ML-DS is a unique disease entity and differs in clinical characteristics and biology from AML in non-DS children. ML-DS is characterized by a low diagnostic white blood cell (WBC) count, young age, FAB M7 morphology, excellent

clinical outcome with survival rates of approximately 80-90% and a high sensitivity to chemotherapy in vivo and *in vitro* [14, 15]. ML-DS is characterized by unique somatic mutations in the *GATA-1* gene, a hematopoietic transcription factor located on the X-chromosome, that result in the truncated protein GATA1s [16]. It is conceivable that additional gene mutations are required for developing ML-DS, as the *GATA1*-mutation (of course together with trisomy 21) is already present in the transient myeloproliferative disorder (TMD) in DS newborns and only 20% of these TMD-patients develop ML-DS in later life [16].

DESIGN AND METHODS

We screened 34 *de novo* ML-DS patients, diagnosed between 1994 and 2007, for the presence of the above mentioned type-I and type-II aberrations and the recently discovered molecular aberrations. Samples were provided by the Dutch Childhood Oncology Group, the AML-'Berlin-Frankfurt-Munster' Study Group, and the Nordic Society for Pediatric Hematology and Oncology. All study groups performed central review of the diagnosis, karyotype and clinical follow-up of the patients. All investigations had been approved by the Institutional Review Board and informed consent was obtained according to local law and regulations. Of the 34 patients, 12 ML-DS patients had a normal karyotype, which is relevant as some of the novel aberrations in non-DS AML are highly associated with a normal karyotype.

Screening of gene mutations was carried out based on availability of material. Mutation analysis was performed for the hotspot regions of the *NPM1, CEPBA, MLL* (i.e. partial tandem duplications (PTD), *WT1*, *FLT3* (i.e. internal tandem duplications (ITD) and tyrosine kinase domain mutations (TKD), *N-RAS* and *K-RAS*, *PTPN11, KIT*, *IDH1/ IDH2*, and the *DNMT3A* genes as previously described [2, 13]. In addition, we investigated the presence of the cryptic translocation *NUP98/NSD1* by reverse transcriptase-polymerase chain reaction (RT-PCR) [7]. A complete list of investigated regions per gene, primers and PCR conditions is provided in Supplementary Table S1. Purified polymerase chain reaction products were bi-directionally sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems Inc., Foster City, CA, USA). The sequence data were assembled and analyzed for mutations using CLC Workbench version 3.5.1 (CLC Bio, Aarhus, Denmark). The 'fusion gene' type-II aberrations, i.e. *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22)/ t(16;16)(p13;q22), t(15;17)(q22;q21) have been described to be mutually exclusive with *NPM1* mutations, *CEBPA* double mutations, and *MLL*-PTD aberrations. Therefore, and as the latter aberrations result in maturation arrest, they were considered type-II aberrations [2]. Mutations in *FLT3, N/K-RAS, PTPN11, KIT*, and *WT1* were considered type-I aberrations [2].

RESULTS AND DISCUSSION

The median age of the ML-DS patients was 1.8 years (range 0.7-2.7 years), the median WBC was 7.1 x109/l (range 3–168 x 109/l) and 42% were male. Patient characteristics of all ML-DS patients are described in detail in Table 1.

In our cohort, only mutations were found in the RAS-pathway; i.e. 2/34 (7%) of the patients carried the G12D mutation in the *K-RAS* gene, and in 1 patient (3%) the G12D mutation in *N-RAS* was found. These *RAS*-mutations have been described in approximately 20% of non-DS pediatric AML patients [2]. In a previous report by Chen *et al*. *N-RAS* mutations were reported in 1 out of 9 ML-DS and 0/11 TMD patients [17]. We did not find any aberrations in *FLT3* or *KIT*, which partly confirms the results of previous smaller series [18, 19]. Shimada *et al.* did not detect any aberrations in *MLL* or *FLT3* [19], whereas Malinge *et al*. detected *FLT3*-mutations in ML-DS in 2/7 patients [18].

Of interest, no *WT1* mutations were found, but 4/34 (17%) ML-DS patients carried the rs16754 single nucleotide polymorphism (SNP) in the *WT1* gene. High *WT1* expression can be detected in both non-DS AML as ML-DS, which is used as a marker for minimal residual disease (MRD) detection in TMD [20] and non-DS pediatric AML [21]. To date, no *WT1* SNPs have been described in ML-DS patients. The *WT1* synonymous rs16754 SNP is proven to be of prognostic influence in adult AML [22] but there is controversy about the prognostic impact in pediatric non-DS AML [23]. Hollink *et al*. did not find any prognostic significance [23], whereas Ho et al identified that this *WT1* SNP as an independent predictor of favourable outcome [24]. In our ML-DS cohort, there were no significant differences neither in survival (p=0.61) nor in clinical characteristics between patients with and without carrying this SNP, although it should be emphasized that patient groups are small. Two patients with the *WT1* rs16754 SNP simultaneously had a *RAS*-mutation.

Mutations in *CEBPA*, *NPM1*, *MLL*-PTD, *DNMT3A* and *IDH1/2* were not found in the 34 patients studied. Two patients (7%) carried the *IDH1* rs11554137 SNP. In non-DS pediatric AML this *IDH1* SNP was found in 47/460 cases (10%) [12]. The *NUP98/NSD1* transcript (present in 16.1 % of non-DS CN AML pediatric patients [7]), was not detected in any of our samples.

When we looked into the expected frequency of the mutations in our ML-DS cohort compared to the non-DS AML pediatric AML patients (calculated from a binomial distribution), only the frequency of *WT1*, *FLT3-ITD*, and *nRAS*- mutations appeared to be significantly different (expected vs. observed), however this may be due to the sample size (n=34). For an overview of the mutations and frequencies see Table 2.

We conclude that the well-known (molecular) type I/ type II aberrations, that are relevant in pediatric non-DS AML, are absent or rare in ML-DS patients. Except for mutations in the *RAS*-gene (and SNPs in the *WT1* and *IDH1* genes), we did not detect any aberrations. Although this is the largest ML-DS cohort screened for molecular aberrations so

far, we may still have missed rare events. Our study underscores the unique signature of ML-DS, and stresses the fact that further research is needed to unravel the molecular abnormalities involved in the leukemogenesis of this specific disease.

Table 1

Characteristics of ML-DS patients

ID	sex	age	WBC	karyotype
1	M	2.5	6	48,XY,t(9;14)(p21;q24),+11,der(16)t(1;16)(q22;p13),+21c[21]/47,XY,+21c[14]
2	M	1.4	6	48,X,ins(Y;5)(q11;?),der(3)t(3;6)(q2?8;?) or ins(3;6)(q2?8;?),-5,del(6)(q1?4q2?4),+21c,+21,+mar[24]
3	F	2.3	49	47,XX,der(9)inv(9)(p24q2?1)del(9)(q2q3),+21c[14]
4	F	1.9	40	47,XX,der(1)t(1;1)(p36;q21),t(5;6)(p15;p23),+21c[23]
5	F	2.4	3	47,XX,r(7)(p22q22).ish r(7)(WCP7+,D7Z1+,D7S486-,164D18-,3K23-),+21c
6	F	1.2	53	47,XX,?del(3)(q?26),add(17)(q?21),add(20)(q11),+21c[10]
7	F	1.4	10	46,XX,dic(15;21)(p11;p11),+21c[10]/46,idem,t(3;5)(q24-q25;p15)[4]/46,idem,t(3;5)(q24-q25;p15),add(10)(p31~32)[6]
8	M	1.8	21	47,XY,del(13)(q13q21),+21c[3]/47,XY,+21c[3]
9	F	1.9	11	47,XX,del(7)(p11.1),+21c[3]/47,XX,+21c[4]
10	M	1.3	6.4	47,XY,t(4;15)(q?21;q?21),del(7)(q?31q?33),+21c/47,XY,+21c
11	F	2.2	7	45-46,XX,der(1)t(1;6)(q31;q?),ins(4;1)(q12;q25q44),-6,-7,der(7)t(6;7)(p21;p22),der(7)t(7;8)(q2?2;q2?3),der(11)t(7;11)(p14;p15),+21c,der(22)t(1;22)(q25;p11)[cp13]/47,XX,+21c[28]
12	F	1.5	7.2	48,XX,+8,+21[5]/47,XX,+21c[18]
13	M	1.0	3.5	47~49,XY,+?19,+21c,+21[cp7]/47,XY,+21c[5]
14	F	2.0	6	48,XX,der(9)t(1;9)(q23;p22),+11,?del(13)(q14q21),+21c[18]
15	M	2.7	4.9	48, XY, +21, +21c
16	F	0.9	3.2	47,XX+21c[13]
17	M	2.3	12	47,XY,+21c [11]
18	F	2.7	4.8	47,XX+21c[6]
19	M	1.1	20	47,XY+21c[15]
20	M	1.3	18	47,XY+21c[1]
21	M	0.9	47	47,XX+21c
22	M	1.8	7	47,XY,+21c [13]
23	M	0.9	4.7	47,XX+21c
24	M	2.0	26	47,XY+21c[25]
25	F	0.8	4.2	47,XX+21c
26	F	1.2	17	47,XX+21c
27	F	1.8	5.9	47,XX+21c
28	M	2.0	26	NA
29	F	2.2	9	NA
30	F	0.9	19	NA
31	F	1.1	26	NA
32	F	2.0	168	NA
33	M	1.3	6	NA
34	F	0.7	4	NA

Age in years; WBC= white blood cell count (*10_9/L); NA= not available

Table 2
Frequency of aberrations in ML-DS patients compared to non-DS AML patients

Genes	AML(n)	Aberrant(n)	Frequency(%)	95% CI interval	Genes	ML-DS(n)	Aberrant(n)	Frequency(%)	Expected frequency (%)	*P*-value
NPM1	337	26	7.7	0.05-0.11	*NPM1*	34	0	0	2.6	0.066
CEBPA	282	17	6.0	0.04- 0.09	*CEBPA*	34	0	0	2.1	0.122
MLLPTD	309	7	2.3	0.01-0.05	*MLLPTD*	27	0	0	0.8	0.538
WT1	330	29	8.8	0.06-0.12	*WT1*	34	0	0	3.0	*0.044**
FLT3_ITD	372	67	18.0	0.14- 0.22	*FLT3_ITD*	34	0	0	6.1	*0.001**
FLT3_TKD	330	9	2.7	0.01- 0.05	*FLT3_TKD*	34	0	0	0.9	0.39
nRAS	353	57	16.1	0.13- 0.20	*nRAS*	34	1	2.9	5.5	*0.003**
kRAS	353	13	3.7	0.02- 0.06	*kRAS*	34	2	5.9	1.3	0.279
PTPN11	350	7	2.0	0.01- 0.04	*PTPN11*	34	0	0	0.7	0.503
KIT	368	31	8.4	0.06- 0.12	*KIT*	34	0	0	2.9	0.051
DNMT3A	140	3	2.1	0.01- 0.06	*DNMT3A*	27	0	0	0.7	0.479
IDH1	281	7	2.5	0.01- 0.05	*IDH1*	27	0	0	0.8	0.424
IDH2	281	9	3.2	0.02- 0.06	*IDH2*	27	0	0	1.1	0.331
NUP98/NSD1	293	13	4.4	0.03- 0.07	*NUP98/NSD1*	27	0	0	1.5	0.297

* indicates a significant difference

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CHAPTER 5

NORMAL KARYOTYPE IS A POOR PROGNOSTIC FACTOR IN MYELOID LEUKEMIA OF DOWN SYNDROME: A RETROSPECTIVE INTERNATIONAL STUDY.

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ABSTRACT

Myeloid leukemia of Down Syndrome has a better prognosis than sporadic pediatric acute myeloid leukemia. Most myeloid leukemia of Down syndrome cases are characterized by additional cytogenetic changes besides the constitutional trisomy 21, but their potential prognostic impact is not known. We therefore conducted an international retrospective study of clinical characteristics, cytogenetics, treatment, and outcome of 451 children with myeloid leukemia of Down syndrome. All karyotypes were centrally reviewed before assigning patients to subgroups.

The overall 7-year event-free survival for the entire cohort was 78% (±2%), with overall survival 79% (±2%), cumulative incidence of relapse 12% (±2%), and cumulative incidence of toxic death 7% (±1%). Outcome estimates showed large differences across the different cytogenetic subgroups. Based on the cumulative incidence of relapse, we could risk-stratify patients into two groups: normal karyotype cases (n=103) with a higher cumulative incidence of relapse (21% (±4%)) than cases with an aberrant karyotype (n=255) with a cumulative incidence of relapse of 9% (±2%) (p=0.004). Multivariate analyses revealed white blood cell counts ≥20 x10^9/l and age >3 years as independent predictors for poor event-free survival event-free survival, while normal karyotype independently predicted inferior overall survival, event-free survival, and relapse-free survival.

In conclusion, this study showed large differences in outcome within myeloid leukemia of Down Syndrome patients and identified novel prognostic groups that predicted clinical outcome and hence may be used for stratification in future treatment protocols.

INTRODUCTION

Children with Down Syndrome (DS) have an increased risk of developing leukemia, including acute myeloid leukemia (AML) and acute lymphoblastic leukemia [1, 2]. These children develop a unique type of AML referred to as Myeloid Leukemia of Down Syndrome (ML-DS), which is recognized as a separate entity in the new WHO-classification [3]. ML-DS is characterized by a low diagnostic white blood cell (WBC) count, myelofibrosis with a low number of leukemic blasts in the marrow [3], mostly FAB M7 morphology, young age at diagnosis (it occurs almost exclusively in children < 5 years), and superior clinical outcome when treated with reduced intensity chemotherapy protocols without stem cell transplantation [4-10]. ML-DS patients have an increased risk of side effects, hence there is a delicate balance between anti-leukemic efficacy and treatment related toxicity. Drug resistance profiles showed that ML-DS blasts are particularly sensitive to various chemotherapeutic drugs *in vivo* and *in vitro* [11, 12], which enables dose reduction.

Somatic mutations in the gene encoding for the transcription factor *GATA1*, localized on the X chromosome (Xp11.2), are pathognomonic for ML-DS [13, 14]. This transcription factor regulates the differentiation of megakaryocytes and erythrocytes. Mutations mainly occur in exon 2 and lead to the truncated protein GATA1s, and are unique for every patient [15, 16].

Age has been recognized as a prognostic factor in ML-DS, with an inferior outcome in the limited number of children aged over 4 years [17]. In fact, it has been proposed that DS children who present over 4 years of age in fact suffer from sporadic AML occurring in a Down syndrome child, rather than 'true' ML-DS [18]. In addition, ML-DS-patients with a history of transient myeloproliferative disease (TMD) had a significantly better outcome than children with ML-DS without documented TMD [19]. Until now, no other prognostic factors have been identified in ML-DS.

The leukemic blasts from the majority of the patients with ML-DS (72%) show additional cytogenetic changes apart from the constitutional trisomy 21 [20]. A previous international-BFM study, performed by Forestier *et al*., showed that the most frequent gains involved chromosomes 8 (27%), 21 (23%), 11 (8.1%), and 19 (7.4%); whereas chromosomes X (3.2%; only females), 5 (1.5%), and 7 (2.2%) were commonly monosomic. The most frequent partial imbalances were duplication 1q (16%), deletion 7p (10%), and deletion 16q (7.4%) [20]. However, the potential clinical impact of these cytogenetic abnormalities is not known and has not been well studied, mainly due to the small patient numbers in individual series [9, 10, 20-22].

In current treatment protocols of non-DS pediatric AML patients, stratification is based on cytogenetics and response to therapy [23]. In ML-DS, no prognostic cytogenetic groups have yet been identified, nor any other prognostic factors allowing a risk-stratified approach.

We therefore conducted a large international study of clinical and outcome data including cytogenetic records from children with ML-DS collected from 13 collaborative study groups. Our aim was to identify differences in outcome related to cytogenetics and clinical characteristics in ML-DS. This was approached by analyzing differences in the cumulative incidence of relapse (CIR), reflecting leukemia resistance, and hence avoiding the influence of toxic (non-leukemic) events on survival estimates. This may result in risk-group stratification and risk-group directed therapy for these patients in the future. In addition, we compared the outcome of the different cytogenetic groups in ML-DS with non-DS AML patients and from the same era treated on AML-BFM regimens as a reference cohort.

METHODS

Patients

Data on 451 patients with ML-DS were collected from 13 collaborative study groups participating in the International AML-BFM Study Group, including the Berlin-Frankfurt-Munster Study Group (Germany and Austria; n=122), the Japanese Pediatric Leukemia/Lymphoma Study Group (Japan; n=96), the Société Française de Lutte contre les Cancers et Leucémies de l'Enfant et de l'Adolescent (France; n=45*)*, the Czech Pediatric Hematology Working Group (Czech Republic; n=6), St. Jude Children's Research Hospital (USA, n=8), the Children's Oncology Group Study, POG 9421 (USA, n=57), the Associazione Italiana di Ematologia ed Oncologia Pediatrica (Italy; n=3), the Nordic Society of Pediatric Haematology and Oncology (NOPHO; Denmark, Finland, Iceland, Norway and Sweden; n=44), the Dutch Childhood Oncology Group (the Netherlands; n=23), the Hong Kong Paediatric Haematology and Oncology Study Group (Hong Kong; n=13), the Polish Paediatric Leukaemia and Lymphoma Study Group (Poland; n= 23), the Israel National Study group for Childhood ALL (Israel; n=6), and the Hungarian Pediatric Oncological Network (Hungary; n=5). For comparison, a reference cohort of non-DS AML patients (n=543) from the same treatment era, kindly provided by the AML-BFM Study Group, was used. This study was approved according to local law and guidelines by the Institutional Review Boards.

ML-DS patients, including those with a constitutional trisomy 21 (96.4%) or unbalanced Robertsonian translocation (3.6%), were identified by the various study groups. Patients were eligible if diagnosed between January 1, 1995 and January 1, 2005. Patients who were not treated with curative intent from diagnosis were excluded. The collected data at diagnosis comprised karyotype (if considered evaluable and centrally reviewed), sex, age, white blood cell count (WBC), hemoglobin, platelet counts, immunophenotypic data and FAB morphology. In addition, we collected data on treatment,

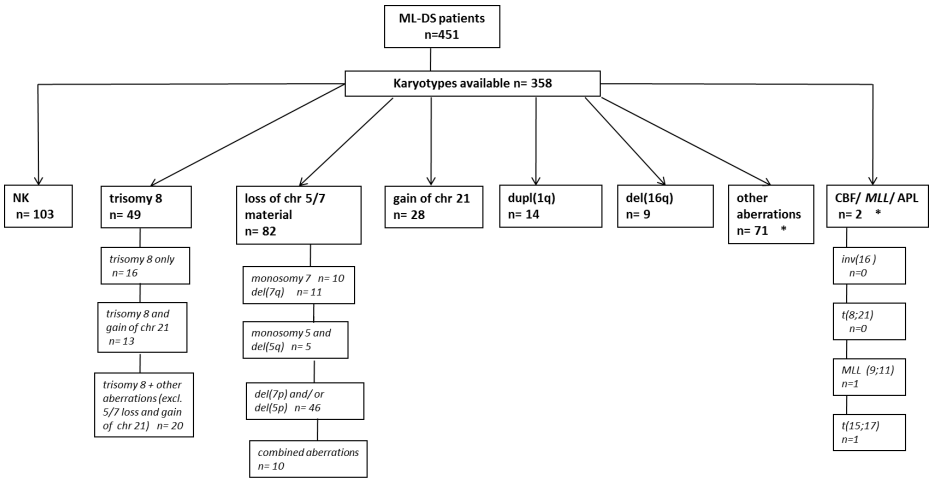
such as therapy protocol (cumulative dosages of drugs), including stem-cell transplantation (SCT), and all events during follow-up (including non-responders, relapse, second malignancy or death). Only patients between 6 months and up to 5 years of age were included in the analyses; TMD-patients were excluded. Patients were treated on national or collaborative group AML trials. The treatment protocols were approved according to local law and guidelines by the Institutional Review Boards of each participating center and/or collaborative group.

Cytogenetic results

All karyotypes were provided after review by a national collaborative group, and centrally reviewed by 2 cytogeneticists (EF, BJ). FISH analyses were not standardly performed. Of the 451 cases, karyotypes were available from 358 (79%), comprising 103 (29%) with a normal karyotype (NK; i.e. with the constitutional trisomy 21 only), 55 (15%) with numerical aberrations only, and 120 (34%) with structural aberrations only. Both types of aberrations were found in 80 karyotypes (22%). Typical nonrandom cytogenetic aberrations, such as t(8;21)(q22;q22) and inv(16)(p13q22), frequently found in non-DS pediatric AML were not identified in the DS patients. Only one case had the acute promyelocytic leukemia-associated t(15;17)(q22;q21) and only one had an *MLL* rearrangement – t(9;11)(p21;q23).

As there was no *a priori* knowledge on the prognostic impact of the various cytogenetic groups in ML-DS, the classification of the cases was based on the premise that all groups should be mutually exclusive, i.e. each patient was included only once, although we could not avoid some overlap in additional cytogenetic abnormalities, and sufficiently large (≥5 cases) to allow meaningful statistical analyses.

The numerically largest group included 103 patients (29%) with a normal karyotype (NK). Another entity that was readily delineated consisted of 49 cases with trisomy 8 (14% of all cases), either as a single abnormality (n=16), or with additional cytogenetic aberrations (n=33). The latter group included a) trisomy 8 and gain of chromosome 21 (n=13, ± other additional changes); and b) trisomy 8 and other changes (n=20, excluding chromosome 5/7 aberrations and excluding +21). Next, a group of 82 cases (23%) with losses of chromosome 5/7 material (excluding those with +21) was distinguished. This group could be further subdivided into 50 cases with abnormalities of the p (short) arms only, 13 cases with monosomies 5/7, 10 cases with del(5q)/del(7q), and 9 cases with changes of both the p and q (long) arms of chromosomes 5/7. Other smaller groups consisted of 28 cases (6%) with a gain of chromosome 21 (in addition to +21c); 14 cases (4%) with a duplication of chromosome 1q; and 9 cases (3%) with a deletion of chromosome 16q. Finally, a group of 73 cases (20%) remained, harboring other aberrations that could not be sub-categorized further (Figure 1) (FigureS1) .



•for analyses in this paper 'CBF/*MLL*/APL ' were included in the 'other aberrations' group
NK= normal karyotype; dupl= duplication; del= deletion

Figure 1
Hierarchy of cytogenetic groups within ML-DS delineated in the present study.

Statistical analyses

Complete remission (CR) was defined as less than 5% blasts in the bone marrow, with regeneration of normal hematopoiesis, and absence of leukemic cells in the cerebrospinal fluid or elsewhere. Patients who failed to achieve CR in time (as specified in the various protocols) were classified as non-responders and considered as failures at day 0. Early death was defined as any death within the first 4-6 weeks of treatment, before evaluation of CR.

Overall survival (OS) was measured from the date of diagnosis to the date of last follow-up or death from any cause. Event-free survival (EFS) was calculated from the date of diagnosis to the first event (non-response, relapse, second malignancy, or death) or to the date of last follow-up. For the OS and EFS analyses, patients who did not experience an event were censored at the time of last follow-up. The Kaplan-Meier method was used to estimate the 7-years probabilities of OS (pOS) and EFS (pEFS), and survival estimates were compared using the log-rank test. Cumulative incidence functions of relapse (with other events and death while in CR as competing event) and cumulative incidence (CI) of toxic death were constructed using the method of Kalbfleisch and Prentice and compared using Gray's test. For multivariate analysis, the Cox proportional-hazard regression model was used. We focused on differences in relapse-free survival (RFS) in order to avoid the influence of non-leukemic events on survival estimates.

Continuous variables were categorized according to cut-off points; age < or ≥ 3 years, WBC counts < or ≥ 20 x 10^9 and Ara-C <or ≥ 20.000 mg/m^2. The $\chi 2$ or Fisher exact test

was used to compare discrete variables among groups; the Mann-Whitney U test was used for continuous variables. All *p*-values are descriptive and explorative, and were considered significant if ≤ 0.05. Statistical analyses were performed using SAS software (SAS-PC, Version 9.1).

RESULTS

Clinical characteristics

The median age of all ML-DS patients (n= 451) was 1.8 years (range 6 months - 5.0 years) and the median WBC count was 7.0 x10^9/l (range 0.8 – 290 x10^9/l). Male - female distribution was almost equal (49.9% vs. 50.1%). Only two (0.5%) patients had CNS involvement. Patient characteristics of the entire cohort are described in detail in Table 1.

Table 1.
Clinical characteristics of the ML-DS patients

	all patients	patients with evaluable kayotypes	*p*- value
N	451	358	
Male sex, n(%)	225 (49.9)	183 (51.0)	0.78
Median age (yrs)	1.8 (0.5-5.0)	1.8 (0.5-5.0)	0.76
< 3 years (%)	399 (91.1)	317 (90.8)	
≥ 3 years (%)	39 (8.9)	32 (9.2)	
Median WBC (x10^9/L)	7.0 (0.8-290)	7.0 (0.8-290)	1.0
< 20 x10^9/L (%)	363 (81.8)	289 (81.9)	
≥ 20 x10^9/L (%)	81 (18.2)	64 (18.1)	
CNS involvement, n(%)	3 (0.7)	2 (0.6)	0.54
Hepatomegaly, n(%)	247 (54.8)	193 (53.8)	0.9
Splenomegaly, n(%)	180 (39.9)	147 (40.9)	0.9

WBC white blood cell count; CNS central nervous system

The median follow-up time of survivors was 4.9 years. Forty-three percent (192 patients) received therapy reduction, or were treated with adjusted Down Syndrome treatment protocols. Outcome parameters did not differ significantly between these groups. Six patients were also treated with irradiation, 3 patients received central nervous system irradiation, whereas the radiation target was not specified for the three other patients.

Ninety-two percent of all patients reached complete remission. The 7-year EFS and OS of all included 451 patients were 78% (± 2%) and 79% (± 2%), respectively. The 7-year

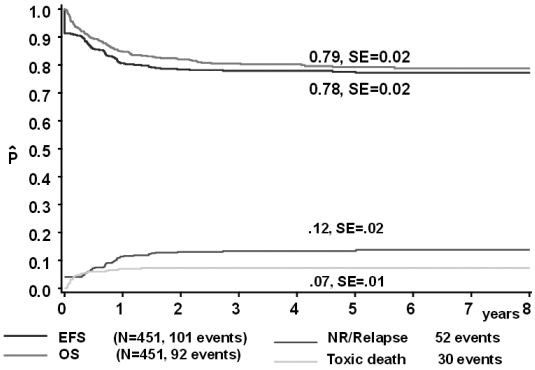


Figure 2: Survival curves of all 451 ML-DS patients included in this study.
The 7-yr overall survival (OS) was 79% (±2%); the 7-yr event-free survival (EFS) 78% (±2%); the 7-yr cumulative incidence of relapse (CIR) was 12% (±2%); and the cumulative incidence (CI) of toxic death at 1.5 years from diagnosis was 7% (±1%). NR= non remitters. (see color figure on page 160)

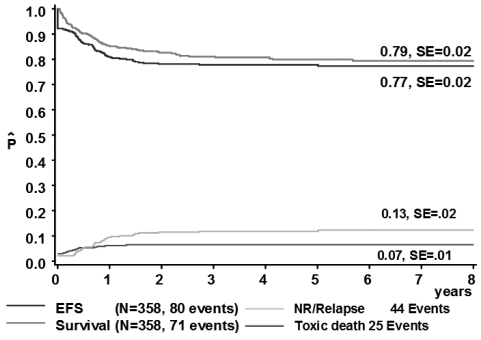


Figure 3: Survival curves of the 358 ML-DS patients with evaluable karyotypes
The 7-yr overall survival (OS) was 79% (±2%); the 7-yr event-free survival (EFS) 77% (±2%); the 7-yr cumulative incidence of relapse (CIR) was 13% (±2%); and the cumulative incidence (CI) of toxic death at 1.5 years from diagnosis was 7% (±1%). NR= non remitters. (see color figure on page 160)

CIR was 12% (± 2%), and cumulative incidence of toxic death was 7% (± 1%) (Figure 2). Of all patients with evaluable karyotypes (n=358), the CR rate was 92%, the 7-year EFS and OS were 77% (± 2%) and79% (± 2%), respectively. The 7-year CIR was 13% (± 2%), and cumulative incidence of toxic death was 7% (±1%) (Figure 3). There were no statistically significant differences between these two groups when comparing various outcome estimates. We therefore conclude that there was no selection bias between the entire study population and the subgroup with informative karyotypes.

In total 25 (5.5%) patients were transplanted in CR1. One patient underwent autologous SCT, 3 patients were transplanted by an allogenic HLA sibling, and 3 patients received a matched family donor transplant; these specifications were not known from

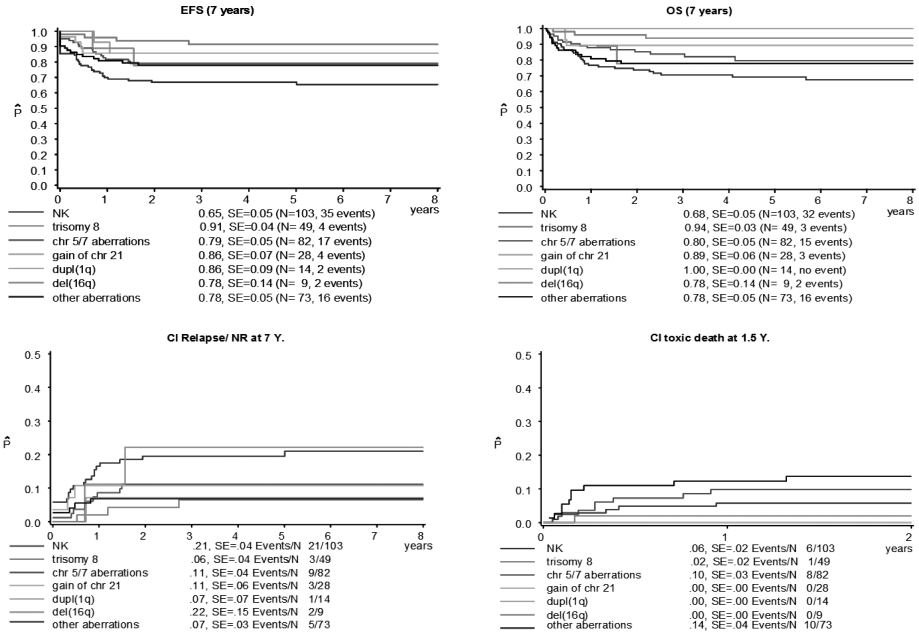


Figure 4

Survival curves for ML-DS patients (n=358) based on their cytogenetic status (see color figure on page 161)

A. Event-free survival curves
B. Overall survival curves
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

Assignment to groups was based on cytogenetic status, as identified after central reviewing.

all the other patients. Forty percent of all transplanted patients died (10/25), half of them due to the leukemia.

Outcome of cytogenetic subgroups

There were no significant differences in the frequency distribution of the various cytogenetic subgroups between the collaborative groups apart from the French cohort, which consisted of a relatively large proportion of NK ML-DS cases. This, however, did not influence the outcome estimates significantly, so there was no studygroup effect in the overall results.

Interestingly, outcome estimates showed large differences across the different cytogenetic subgroups (Figure 4). An overview of all outcome estimates per subgroup is given in Table 2.

Based on the CIR estimates, patients could be divided into groups with a high CIR (> 20%): comprising NK and del(16q) (n=112), and a low CIR (< 20%), comprising all other patients (n=246). Since the first group mainly consisted of NK cases (92%), with only 9

Table 2.
Survival estimates per cytogenetic subgroup

Survival estimates per cytogenetic subgroup	7-yrs CIR	7-yrs OS	7-yrs EFS	7-yrs CI of toxic death
NK (n=103)	21% (+/- 4%)	68% (+/- 5%)	65% (+/- 5%)	6% (+/- 2%)
del(16q) (n=9)	22% (+/- 15%)	78% (+/- 14%)	78% (+/- 14%)	0%
loss of chromosome 5/7 material (n=82)	11% (+/- 4%)	80% (+/- 6%)	79% (+/- 5%)	10% (+/- 3%)
gain of chromosome 21 (n=28)	11% (+/- 6%)	89% (+/- 6%)	86% (+/- 7%)	0%
dupl (1q) (n=14)	7% (+/- 7%)	100%	86% (+/- 9%)	0%
trisomy 8 (n=49)	6% (+/- 4%)	94% (+/- 4%)	91% (+/- 4%)	2% (+/- 2%)
other aberrations (n=73)	7% (+/- 3%)	78% (+/- 5%)	78% (+/- 5%)	14% (+/- 4%)

CIR= cumulative incidence of relape; OS= overall survival; EFS= event-free survival; CI of toxic death= cumulative incidence of toxic death
NK = normal karyotype; del= deletion; dupl= duplication

Table 3.
Clinical characteristics of the NK ML-DS vs. all other cases (with aberrant karyotypes)

	NK	other	*p*- value
N	103	255	
Male sex, n(%)	53 (51.4)	125 (50.8)	0.86
Median age (yrs)	1.7 (0.5-5.0)	1.8 (0.5-5.0)	0.9
< 3 years (%)	96 (93.2)	228 (91.2)	
≥ 3 years (%)	7 (6.8)	22 (8.8)	
Median WBC (x10^9/L)	7.5 (0.8-160)	6.9 (1.5-290)	0.7
< 20 x10^9/L (%)	81 (79.4)	205 (82.7)	
≥ 20 x10^9/L (%)	21 (20.6)	43 (17.3)	
CNS involvement, n(%)	1 (0.01)	1 (0.01)	0.35
Hepatomegaly, n(%)	56 (54.9)	132 (55.0)	0.97
Splenomegaly, n(%)	47 (46.1)	95 (39.6)	0.23

WBC white blood cell count; CNS central nervous system

cases with del(16q) with two events, we decided to perform further analyses comparing the NK cases (29%) with all cases with aberrant karyotypes (71%). Clinical characteristics did not differ between these two groups (Table 3). The rate of complete remission was significantly lower in NK ML-DS compared to the aberrant kayotype cases (87% vs. 96%; $p< 0.01$). The NK patients had significantly worse survival outcomes: 7-yr CIR of 21% (±

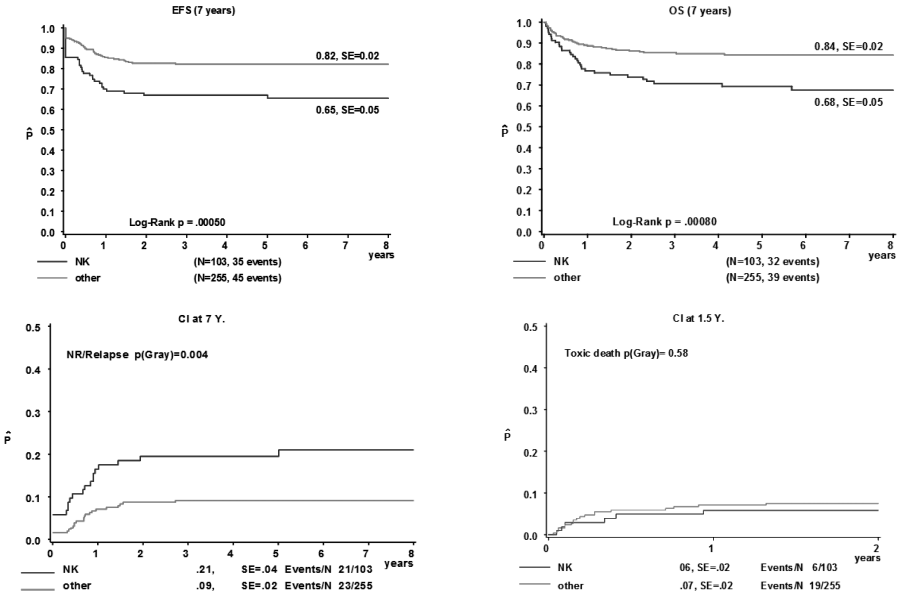


Figure 5

Survival curves for NK ML-DS patients (n=103) versus all other patients (n=255). (see color figure on page 162)

A. Event-free survival curves
B. Overall survival curves
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

Assignment to groups was based on cytogenetic status, as identified after central reviewing.

4%) vs. 9% (± 2%) (p=0.004), 7-yr OS of 68% (± 5%) vs. 84% (± 2%) (p=0.0008), and 7-yr EFS of 65% (± 2%) vs. 82% (± 5%) (p=0.0005). The CI of toxic death was not significantly different between NK ML-DS and patients with aberrant karyotypes: 6% (± 2%) vs. 7% (± 2%) (p= 0.58) (Figure 5). Regarding the rate of complete remission, NK-patients did reach significantly less remission than patients with aberrant karyotypes (87% vs. 96%; p< 0.01)**.**

Chromosome 7 aberrations

Given the presence of a large number of cases with chromosome 7 abnormalities in our ML-DS cohort and the specific prognostic relevance of chromosome 7 abnormalities in non-DS AML we focused on this group separately [24]. ML-DS patients with chromosome 7 aberrations did not have significantly different survival parameters compared to all other patients (P=0.63). This group was further subdivided into cases with a monosomy 7 (n=10) and those with a del(7q) (n=11). Patients with monosomy 7 tended to have worse survival estimates than patients with a del(7q), but this was not statistically sig-

nificant: 7-yr EFS 67% (± 14%) vs. 81% (± 10%) (p= 0.40), 7-yr OS 59% (± 17%) vs. 88% (± 8%) (p= 0.2), and 7-yr CIR 9% (± 9%); vs. 20% (± 14%); (p=0.36) (Figure S2).

Regarding the 5 patients with chromosome 5 aberrations, 4 of them were alive after at least 4 years of follow up, although one of them suffered from severe infections during treatment. One of them died 2 months after diagnosis due to sepsis in induction, this patient also had a congenital heart defect.

Other prognostic factors

Patients with high WBC counts (≥ 20 x10^9/l) tended to have a worse 7-yr EFS than patients with a lower WBC count (< 20 x10^9/l): 79% (± 2%) vs. 70% (± 5%); p=0.047). However, this did not translate into a significant difference for 7-yr OS (80% (± 2%) vs. 73% (± 5%); p=0.07). This was due to the occurrence of events in the induction phase; the CR rate was significantly lower in patients with a high WBC counts (93% vs. 81%; p=0.007). The 7-yr CI of toxic death and CIR did not differ significantly: CI toxic death 6% (± 3%) vs. 7% (± 1%) (p=0.83) and CIR 16% (± 4%) vs. 10% (± 2%) (p=0.1) (Figure S3).

In addition, after evaluating various cut-off points for age, patients aged <3 years, had a significantly better 7-yr EFS and CIR than patients aged ≥ 3 years (EFS 78% (± 2%) vs. 65% (± 7%) (p=0.04) and CIR 11% (± 2%) vs. 21% (± 6%) (p=0.05) (Figure S4). This was also due to events in induction, with a higher borderline statistically significant CR rate for patients aged <3 years (93% vs. 84%; p=0.08). The CI of toxic death was not significantly different between these 2 age groups (7% (± 1%) vs. 5% (± 3%)(p=0.58)), nor was OS (80% (± 2%) vs. 69% (± 7%)(p= 0.10)).

Immunophenotyping

ML-DS cases positive for the lymphoid co-expression marker CD7 (n=187/221) had a borderline better EFS (79% (± 3%) vs. 64% (± 8%); p=0.054) (Figure S5). However, no significant differences were seen for OS, CIR or CI of toxic death. Expression of CD56 (neural cell adhesion molecule) (n=92/169) was not significantly associated with any of the outcome estimates (Figure S6), whereas CD34 (expressed on early hematopoietic cells) positive cases (n=94/221) had a worse EFS (70% (± 5%) vs. 82% (± 3%); p=0.049) and a higher CIR (16 ± 4% vs. 7 ± 2%; p=0.04) than CD34- negative cases (Figure S7).

Treatment

No significant differences in outcome estimates, CIR and CI of toxic death were seen between groups treated with different cumulative dosages of anthracyclines and etoposide. Patients treated with higher cumulative dosages of cytarabine (≥20 g/m^2) had a significantly better 7-yr EFS (84% (± 3%) vs. 75% (± 3%); p=0.043) and a trend towards a better 7-yr OS (85% (± 3%) vs. 77% (± 3%); p=0.056) than patients treated with lower doses (<20 g/m^2) (Figure S8). There was also a trend for lower 7-yr CIR in patients

treated with higher doses (7% (± 2%) vs. 14% (± 2%); p=0.06). The CI of toxic death was significantly lower in the patients treated with higher cytarabine doses (≥ 20 g/m^2) (2% (± 1%) vs. 9% (± 2%); p=0.02).

Forty-three percent of all patients received therapy reduction or were treated with adjusted DS treatment protocols. Overall, no differences in outcome estimates were found between patients treated with therapy reduction and those who received standard therapy.

Table 4.
Multivariate analysis of survival parameters of survival of ML-DS patients

Outcome	Variable	Hazard ratio (HR)	95% confidence interval (CI)	*p*- value
OS	age ≥ 3 years	1.71	0.95 - 3.08	0.07
	WBC ≥ 20.000	1.56	0.96 - 2.52	0.07
	NK	1.53	0.99 - 2.52	0.05
EFS	age ≥ 3 years	1.92	1.10 - 3.33	0.02
	WBC ≥ 20.000	1.61	1.01 - 2.56	0.04
	NK	1.65	1.05 - 2.59	0.03
RFS	age ≥ 3 years	2.55	1.23 - 5.28	0.01
	WBC ≥ 20.000	1.83	0.97 - 3.46	0.06
	NK	2.22	1.19 - 4.13	0.01

OS overall survival; EFS event-free survival; RFS relapse-free survival; NK normal karyotype

Multivariate analyses

Cox regression analysis of survival estimates from diagnosis revealed both age ≥3 years and WBC counts ≥20x10^9/l as independent predictors for poor EFS (see Table 4), but not for OS. In addition, NK independently predicted for poor OS (hazard ratio [HR]= 1.53 and *p*=0.05), EFS (HR= 1.65; *p*=0.03) and for RFS (HR= 2.22; *p*=0.01). Age ≥3 years was also an independent predictor for a lower RFS with a (HR= 2.55; *p*=0.01).

DISCUSSION

In this collaborative study we analyzed a large international series of ML-DS cases, with the aim to identify differences in outcome related to cytogenetic features that may result in risk group stratification and risk-adapted therapy for ML-DS patients in the future. The results underscore the importance of international collaboration in the investigation of rare diseases or groups.

It was confirmed that overall outcome for ML-DS was superior to AML in non-DS children, with a 7-yr OS and 7-yr EFS of 78% and 79% respectively in ML-DS, compared

to 62% and 50% for non-DS AML patients from the same era treated on AML-BFM regimens as a reference cohort (both $p<0.001$; see Figure S9). Of interest, the OS and EFS estimates were superimposed in ML-DS, suggesting that most relapsed patients could not be salvaged. However, it is unknown whether these patients were treated with curative intent at relapse. Although there is great concern for toxic mortality in ML-DS, in the present series relapse was more frequent than treatment related mortality, with cumulative incidences of 12% and 7%, respectively. The relapse frequency of 12% is remarkably low when compared with non-DS AML patients from the AML-BFM study group from the same era, who had a CIR of 42% ($p<0.001$). However, the CI of toxic death was similar between DS and non-DS children: 7% and 5%, respectively ($p=0.12$). The reasonable balance between toxic death and leukemia relapse in ML-DS may be due to the fact that treatment reduction was frequently applied, as compared to older studies which have reported higher toxic death rates in ML-DS [6, 7, 25].

Non-random cytogenetic aberrations that are common in non-DS pediatric AML, such as core-binding factor (CBF; t(8;21)), *MLL*-rearrangements and t(15;17) were identified in single cases only in our ML-DS cohort, which is in line with previous studies [20].

The salient finding in the present study was that NK ML-DS patients had poor survival parameters compared to ML-DS cases with aberrant karyotypes, and that NK independently predicted for poor clinical outcome. Therefore, NK may be used for treatment stratification in future treatment ML-DS protocols. In the NK ML-DS cases, the complete remission rate was significantly lower, and relapse (CIR 21%) determined prognosis to a greater extent than cumulative toxic death (6%). Hence, in this subgroup no further therapy reduction should be applied, whereas until now the increase in survival in ML-DS patients has mainly been achieved through the application of reduced-intensity chemotherapy protocols [4, 6, 8, 9]. In fact, treatment intensification may even be needed. In order to reduce the number of induction failures a double induction for instance based on day 15 bone marrow blasts may be considered in patients with residual demonstrable leukemia. In addition, detection of *GATA1*-mutations using real-time quantative PCR may be feasible as a marker for minimal residual disease (MRD) in the nearby future [26], but is not routinely used yet. Alternative methods for MRD- detection include flowcytometry or reverse-transcription PCR for the *WT1* gene [27]. Increasing the cumulative doses of e.g. cytarabine may be of benefit during consolidation and intensification, as the CIR was los lower in patients treated with higer doses. Recently, stem cell transplantation in ML-DS was reviewed but transplant-related mortality (24%) was significantly higher than for non-DS AML [28], so its use should be limited to cases who do not attain sufficient remission or as salvage therapy at relapse.

Understanding the underlying biology of NK ML-DS may reveal potential new treatment targets. Non-DS pediatric NK AML cases are characterized by various abnormalities, including overexpression of specific genes (*MN1*, *BAALC,* and *ERG*) [29], but also single

gene mutations such as *FLT3*/ITD, *WT1*, *NPM1*, *CEBPA* [30-32], as well as cryptic translocations [33]. We recently showed that the abnormalities mentioned above are absent or rare in (NK-) ML-DS [34]. Hence, the underlying biology of NK ML-DS needs to be studied in more detail, e.g. using novel techniques such as whole genome sequencing.

Non-DS pediatric AML with a trisomy 8 is classified as an intermediate risk group [35]. In the present study, we showed significantly better outcome estimates for the trisomy 8 ML-DS patients in a direct comparison to non-DS AML trisomy 8 patients (CIR of 6% vs. 62%; $p<0.0001$) (Figure S10). Apparently, an additional copy of chromosome 8 has biologically different consequences in ML-DS compared to non-DS AML.

Monosomy 7 is known to be a poor prognostic factor in non-DS pediatric AML, as reported by another I-BFM collaborative study [35]. Outcome was significantly worse in patients with a loss of the whole chromosome (monosomy 7) than in patients with a del(7q) [24, 35]. In our ML-DS series, such differences were not observed, but numbers were small. Comparing ML-DS and non-DS AML patients revealed that ML-DS patients with monosomy 7 and/or del(7q) had a remarkably lower CIR (14% vs. 52%; $P=0.003$) (Figure S11). Thus chromosome 7 aberrations do not seem to have the same implications in ML-DS as in non-DS pediatric AML.

Interestingly, most chromosome 5/7 losses in ML-DS involved the p-arms rather than the q-arms. This is in contrast to non-DS AML, in which 5q and 7q losses are much more common and also prognostically relevant [35].

Regarding the treatment of ML-DS patients, we have no clear explanation for the fact that the CI of toxic death was significantly lower in the patients treated with higher doses of cytarabine. A hypothesis could be that due to concern for toxicity these patients received different and more intensive supportive care. We did not find any differences in outcome estimates between ML-DS patients treated with therapy reduction and those who received standard therapy, although it needs to be mentioned that no exact details of treatment reduction and individual treatment protocols nor of protocol adherence or individual adaptations of therapy were available due to the retrospective nature of this study.

In terms of prognostic factors other than cytogenetics, Klusmann et al. reported that ML-DS patients with a history of TMD had a significantly better outcome than children with ML-DS without documented TMD [19], but unfortunately we were not able to collect data on whether ML-DS was preceded by TMD.

Age ≥ 3 years and high WBC (>20 x10^9) were identified in the present study as independent predictors for poor outcome (EFS) in ML-DS, which is in concordance with previous studies (9). This is mainly explained by the fact that there is a low(er) complete remission rate in these groups. In addition, these variables are also known from non-DS pediatric AML studies, in which older age and high WBC predict for poor outcome [14]. Regarding age in ML-DS, it has been proposed that DS children who present over 4

years of age in fact suffer from sporadic AML occurring in a Down syndrome child, rather than 'true' ML-DS [18]. For this reason we used the age cut-off in our inclusion criteria, to avoid 'contamination' with non-*GATA1* mutated AML case in Down syndrome children. In addition, AML in children with Down syndrome older than 4 years of age is exceedingly rare [18].

A limitation of this collaborative study is that there was a wide variation in treatment intensity. Although all included patients were treated on collaborative treatment protocols, almost half of the patients received therapy according to protocols or risk arms specifically designed for DS patients and/ or treatment reductions were made in standard protocols. These factors may have biased the study results.

In conclusion, this study showed that NK predicts for poor clinical outcome in ML-DS. As the incidence of relapse is higher than treatment related mortality in these cases, further therapy reduction is not indicated in this group; in fact treatment intensification may be needed. On the other hand, treatment reduction may be feasible in ML-DS cases with aberrant karyotypes. Such treatment stratifications have to be confirmed in prospective clinical studies. As the prognosis of high-risk NK ML-DS patients cannot be explained by the presence of known mutations in non-DS NK AML, the biological background has to be elucidated to identify potential novel targets for therapy.

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CHAPTER 6

GENE EXPRESSION PROFILING IN TRANSIENT MYELOPROLIFERATIVE DISORDER AND MYELOID LEUKEMIA OF DOWN SYNDROME

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ABSTRACT

Children with Down Syndrome have an increased risk of developing myeloid leukemia (ML-DS) that is often preceded by transient myeloproliferative disease (TMD) in newborns, which mostly resolves spontaneously. As both are characterized by mutations in *GATA1*, additional factors are required for progression of TMD into ML-DS.

Affymetrix HumanGenome U133plus2.0 microarrays were used to generate gene expression profiles (GEP) of 14 ML-DS and 10 TMD cases (unpaired). We identified specific signatures for TMD and ML-DS with significant *MLLT3* and *ITGA4* overexpression in ML-DS. *MLLT3* overexpression was not due to mutations in the gene. Functional studies did not show significant differences in proliferation or apoptosis for *MLLT3*-silenced cells using an ML-DS cell-line. However, *IGF1R*, known to be upregulated in ML-DS and contributing to leukemogenesis, was downregulated after *MLLT3* silencing. This may indicate that *MLLT3* plays a role in the regulation of *IGF1R* induced leukemogenesis.

By using unsupervised clustering, all ML-DS cases clustered together and separate from non-DS AML (n=297), hence ML-DS is a unique disease given its strong discriminative GEP.

In conclusion, we found overexpression of *MLLT3* and *ITGA4* in ML-DS compared to TMD and downregulation of *IGF1R* after *MLLT3* in ML-DS silencing. Further investigation for the role of *MLLT3* and *IGF1R* in the evolution from TMD into ML-DS is indicated.

INTRODUCTION

Children with Down Syndrome (DS) have an increased risk of developing leukemia, including both acute myeloid (ML-DS) and acute lymphoblastic leukemia [1, 2]. These leukemias differ in clinical characteristics and biology from leukemias in non-DS children. ML-DS is characterized by a low white blood cell count (WBC), young age, FAB M7 morphology, survival rates of more than 90%, and a high sensitivity to chemotherapy [3, 4]. Reduced treatment intensity in ML-DS does not lead to increased relapse rates, and decreases treatment related mortality [5], leading to increased overall survival.

Previous studies showed that pediatric and adult AML can be accurately classified into cytogenetically distinct subtypes based on their micro-array gene expression profiles (GEP) [6, 7]. In the current study we performed unsupervised clustering (USC) to see whether ML-DS cases cluster separately from e.g. non-DS FAB M7 leukemias in a cohort of non-DS pediatric AML cases.

ML-DS can be preceded by transient myeloproliferative disease (TMD) in newborns, which in most cases resolves spontaneously. Approximately 20% of TMD- patients develop ML-DS later in life [8, 9]. Both TMD and ML-DS blasts are characterized by unique, somatic mutations in the GATA binding protein 1 *(GATA1)* gene, a hematopoietic transcription factor, localized on the X-chromosome, which result in the exclusive expression of a truncated protein GATA1s [10, 11]. As *GATA1*- mutations occur both in TMD and ML-DS [11], and only 20% of the TMD-patients eventually develop ML-DS, additional events are needed for the progression of TMD to ML-DS [8].

Several recent studies provided more insight into the underlying biology of these diseases. For instance, Ge *et al.* reported several overexpressed megakaryopoiesis-related genes encoded on chromosome 21 disturbing normal hematopoiesis [12]. In addition, miR125-b-2, also located on chromosome 21, has been described as a potential oncomiR in both these diseases, as this miR functions in increasing the proliferation and self-renewal of megakaryocytic progenitors [13]. Klusmann *et al.* showed that constitutive activation of the insulin-like growth factor (IGF) signaling pathway via the *IGF1 receptor* (IGF1R) contributes to the survival and proliferation of ML-DS cell lines. This mitogenic pathway converges with mutated *GATA1s* in fetal megakaryopoiesis and leukemogenesis [14].

Previous studies analyzing gene expression profiles either used different platforms (with less probesets), or samples were not purified and contained low or variable blast percentages, which hampers the interpretation of the results [15, 16]; McElwaine et al). To get more insight in the differentially expressed genes involved in the evolution from TMD into ML-DS, we used gene expression profiling with the Affymetrix Human Genome U133 plus 2.0 platform (containing > 54.000 probe sets) including only samples with >80% blasts.

MATERIAL AND METHODS

Patients

Viably frozen bone marrow or peripheral blood samples from 296 de novo pediatric non-DS AML patients [17], 18 ML-DS and 13 TMD cases were provided by the Dutch Childhood Oncology Group (DCOG), the 'Berlin-Frankfurt-Münster' AML Study Group (AML-BFM-SG), the Czech Pediatric Hematology, the St. Louis Hospital in Paris, France, and the Nordic Society for Pediatric Hematology and Oncology (NOPHO). TMD and ML-DS samples were unpaired.

Informed consent was obtained from all patients, after Institutional Review Board approval according to national law and regulations. Leukemic cells were isolated and purified as previously described [18]. All resulting samples contained 80% or more leukemic cells, confirmed morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospin slides after thawing. Cells were lysed in Trizol reagent (Gibco BRL/Life Technologies, Breda, The Netherlands) and stored at -80°C. Genomic DNA and total cellular RNA were isolated according to the manufacturer's protocol, with minor modifications [18]. Studies were carried out based on availability of material (Table 1).

Microarray- based gene expression profiling

RNA integrity was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA, USA). cDNA and biotinylated cRNA was synthesized and hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines. Data-acquisition was performed using 'expresso' (Bioconductor package 'Affy') and probe-set intensities were normalized using the variance stabilization normalization procedure (Bioconductor package 'VSN') in the statistical data analysis environment R, version 2.2.0. [19, 20].

To find signatures for TMD (n=10) and ML-DS (n=14), an empirical Bayes linear regression model was used to compare samples from these groups to each other [21]. Moderated T-statistics p-values were corrected for multiple testing using the False Discovery Rate (FDR) method defined by Benjamini and Hochberg. Supervised clustering and principal component analyses were performed using GeneMath XT 1.6.1 software (Applied Maths, Austin, USA) and GenePattern [22]. Unsupervised clustering analyses was performed and visualized as previously described [7, 23].

Cell culture

The CMK-cell line, derived from a 10-month old boy with ML-DS (DSMZ, Braunschweig, Germany), was cultured in RPMI-1640 medium (Invitrogen, Life Technologies, Breda, The Netherlands) supplemented with 20% fetal calf serum (FCS) (Integro, Zaandam, The Netherlands), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.125 µg/mL fungizone

Table 1: Clinical and genetic characteristics of the TMD and ML-DS patients

ID	disease	sex	age	WBC	karyotype	*GATA-1* mutation	GEP study	Taqman cohort
1	TMD	f	2.0	193	47,XX,+21c	del 2 bp	yes	yes
2	TMD	m	1.0	35	NA	ins 14 bp	yes	yes
3	TMD	f	46.0	75	NA	pointmutation	yes	no
4	TMD	f	1.0	173	NA	del 1 bp, ins 2 bp	yes	no
5	TMD	m	8.0	NA	NA	del 2bp	yes	yes
6	TMD	m	1.0	410	NA	ins 14 bp	yes	yes
7	TMD	m	4.0	NA	NA	pointmutation	yes	yes
8	TMD	f	NA	NA	NA	NA	yes	yes
9	TMD	m	4.0	111	47,XY,+21c	ins 3 bp	yes	no
10	TMD	f	3.0	88	49,XX,+8,+21c,+21	NA	yes	yes
11	TMD	m	3.0	101	47,XY,+21c	del 5bp	no	yes
12	TMD	m	5.0	41	NA	ins 1 bp	no	yes
13	TMD	m	1.0	106	NA	ins 13 bp, del 21 bp	no	yes
14	ML-DS	m	2.0	26	NA	del 2 bp	yes	yes
15	ML-DS	f	2.2	9	NA	del 2 bp	yes	yes
16	ML-DS	m	2.3	12	NA	ins 14 bp	yes	yes
17	ML-DS	m	2.5	6	48,XY,t(9;14)(p21;q24),+11,der(16)t(1;16)(q22;p13),+21c[21]/47,XY,+21c[14]	del 7 bp	yes	yes
18	ML-DS	f	0.9	19	NA	del 5 bp	yes	yes
19	ML-DS	m	1.4	6	48,X,ins(Y;5)(q11;?),der(3)t(3;6)(q2?8;?) or ins(3;6)(q2?8;?),-5,del(6)(q1?4q2?4),+21c,+21,+mar[24]	ins 4 bp	yes	yes
20	ML-DS	f	2.3	49	47,XX,der(9)inv(9)(p24q2?1)del(9)(q2q3),+21c[14]	del 3 bp	yes	yes
21	ML-DS	f	1.9	40	47,XX,der(1)t(1;1)(p36;q21),t(5;6)(p15;p23),+21c[23]	del 6 bp	yes	yes
22	ML-DS	f	2.4	3	47,XX,r(7)(p22q22).ish r(7)(WCP7+,D7Z1+,D7S486-,164D18-,3K23-),+21c	pointmutation	yes	yes
23	ML-DS	f	1.2	53	47,XX,?del(3)(q?26),add(17)(q?21),add(20)(q11),+21c[10]	ins 1 bp	yes	no

Table 1: Clinical and genetic characteristics of the TMD and ML-DS patients (continued)

ID	disease	sex	age	WBC	karyotype	*GATA-1* mutation	GEP study	Taqman cohort
24	ML-DS	f	1.4	10	46,XX,dic(15;21)(p11;p11),+21c[10]/46,idem,t(3;5)(q24-q25;p15)[4]/46,idem,t(3;5)(q24-q25;p15),add(10)(p31~32)[6]	del 2 bp	yes	yes
25	ML-DS	f	1.1	26	NA	del 13 bp	yes	yes
26	ML-DS	m	1.8	21	47,XY,del(13)(q13q21),+21c[3]/47,XY,+21c[3]	ins 1 bp	yes	No
27	ML-DS	f	2.0	6	48,XX,der(9)t(1;9)(q23;p22),+11,?del(13)(q14q21),+21c[18]	ins 1 bp	yes	Yes
28	ML-DS	f	2.0	168	NA	pointmutation	no	Yes
29	ML-DS	f	1.9	11	47,XX,del(7)(p11.1),+21c[3]/47,XX,+21c[4]	del 1bp	no	Yes
30	ML-DS	m	1.8	7	47,XY,+21c [13]	ins 4 bp	no	Yes
31	ML-DS	m	1.3	6	NA	ins 13 bp, del 21 bp	no	Yes
32	ML-DS	m	2.1	9	NA	NA	no	No

MLDS= myeloid leukemia of Down syndrome; TMD= transient myeloproliferative disorder; WBC= white blood cell count (*10_9/L). NA = not available.
NA= not available. Age in years, except for TMD (days).

(Invitrogen) and grown as suspension cultures at 37°C in humidified air containing 5% CO2.

PCR for *MLL-MLLT3* (*AF9*)

All TMD and ML-DS samples were screened for the presence of the fusion product *MLL-MLLT3* (myeloid/lymphoid or mixed-lineage leukemia, translocated to 3 also known as *AF9*) by using Reverse Transcriptase PCR. Primer sequences are described in Supplementary Table S1.

Real-time quantitative PCR (RQ-PCR)

RNA expression levels of the genes of interest (including *MLLT3, ITGA4* (integrin alpha-4*), IGF1R*) were quantified by real-time quantitative RT-PCR (RQ-PCR) in 10 TMD and 16 ML-DS samples. The relative expression was calculated using the comparative cycle time (ΔCt) method, with *GAPDH* as the house-keeping gene [24]. Primer sequences are described in Supplementary Table S2 and S3.

Mutation analysis

All TMD and ML-DS samples were screened for *GATA-1* and *MLLT3* mutations. *MLLT3* mutations were screened for as the expression of this gene was significantly different between TMD and ML-DS patients. For these analyses, exons 2 and 3 of *GATA-1* and all exons (1-10) of *MLLT3* were PCR amplified. Purified PCR products were bi-directionally sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems Inc., Foster City, CA, USA). The sequence data were assembled and analyzed for mutations using CLC Workbench version 3.5.1 (CLC Bio, Aarhus, Denmark).

Protein extraction and Western blot analysis

For Western blot, cell pellets of the CMK cell line, which were stored at −80°C, were quickly thawed and resuspended in 100 µL of lysis buffer composed of 25mM Tris (tris[hydroxymethyl]aminomethane) buffer, 150mM NaCl, 5mM EDTA (ethyl-nediaminetetraacetic acid), 10% glycerol, 1% Triton X-100, 10mM sodium pyrophosphate, 1mM sodium orthovanadate, 10mM glycerolphosphate, 1mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin (Sigma), 10mM sodium fluoride, and 20 µL of freshly prepared sodium pervanadate. Subsequently, cell lysis was allowed for 30 minutes on ice. Cell lysates were cleared by centrifugation for 15 minutes at 10 000*g* (13 000 rpm) and 4°C. Protein concentration was determined by use of the bicinchoninic acid protein assay (Pierce Biotechnology) with different concentrations of bovine serum albumin as standards. Cell lysates containing 20 ng of protein were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell). Western Blots were probed with rabbit monoclonal IgG anti-MLLT3 (Abcam, San

Fransisco, CA, USA) and mouse antibeta-actin (ab6276; Abcam) antisera. Subsequently, the blots were labeled with peroxidase-conjugated antigoat antibody (sc-2020; Santa Cruz Biotechnology) or antimouse antibody (DAKO, Glostrup, Denmark). Chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology) was used to detect luminescence using the Syngene chemigenius (Syngene).

Transfection

The CMK-cell line (DSMZ, Braunschweig, Germany) was cultured in RPMI-1640 medium (Invitrogen) supplemented with 20% fetal calf serum (FCS) (Integro), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.125 µg/mL fungizone (Invitrogen) and grown as suspension cultures at 37°C in humidified air containing 5% CO2.

CMK cells ($10x10^6$) were transfected by electroporation in 400 ul RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) containing 10ul of either the *MLLT3* siRNA or non-target (NT) siRNA (Dharmacon RNAi technologies, Thermo Scientific, Lafayette, CO, USA) as empty control, in 4 mm electroporation cuvettes (BioRad, Hercules, CA, USA). To compensate for the amount of cell death induced merely as a consequence of the electroporation procedure, control cells were electroporated in the absence of any siRNA. Electroporation was performed using an EPI 2500 gene pulser (Fischer, Heidelberg, Germany) applying a square pulse of 350 V for 10 ms. After incubating for 20 min at room temperature, the cells were diluted in 20 ml RPMI 1640 supplemented with 20% FCS (Integro), 100 IU/ml penicillin, 100 ug/ml streptomycin and 0.125 ug/ml fungizone (Invitrogen) and incubated at 37°C and 5% CO2.

Cell viability was assessed by Annexin V/PI staining determined by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

BrdU cell-cycle proliferation assay

CMK ($1x10^6$) were incubated at several timepoints after transfection (with *MLLT3* siRNAs or NT siRNAs) with 10uM BrdU for 1 hour. Cells from the same population without BrdU were used as negative staining control. Next, cells were fixed, treated with DNase to expose BrdU epitopes, incubated with FITC-conjugated anti-BrdU antibodies, and total DNA was stained with 7-AAD following instruction of the manual (FITC BrdU Flow Kit, BD Pharmingen, San Diego, CA, USA). The flow cytometry assay was performed using a FACSCalibur.

Statistics

To find signatures for the different subtypes an empirical Bayes linear regression model was used to compare samples from each group to all other samples [21]. Moderated T-statistics p-values were corrected for multiple testing using the False Discovery Rate (FDR) method defined by Benjamini and Hochberg [25].

Software

R (version 2.2.0 and 2.5.0) and the R packages affy, vsn, e1071, globaltest, limma, multtest and marray were used to run the above-mentioned analyses [19, 21, 26-30]. Hierarchical clustering analysis was performed in Genemaths XT (Applied Maths, Austin, Texas, USA).

RESULTS

Patients characteristics

Samples were available from 13 TMD and 18 ML-DS patients. TMD and ML-DS samples were unpaired. The TMD- patients had a median age of 3.0 days at diagnosis (range 1-46

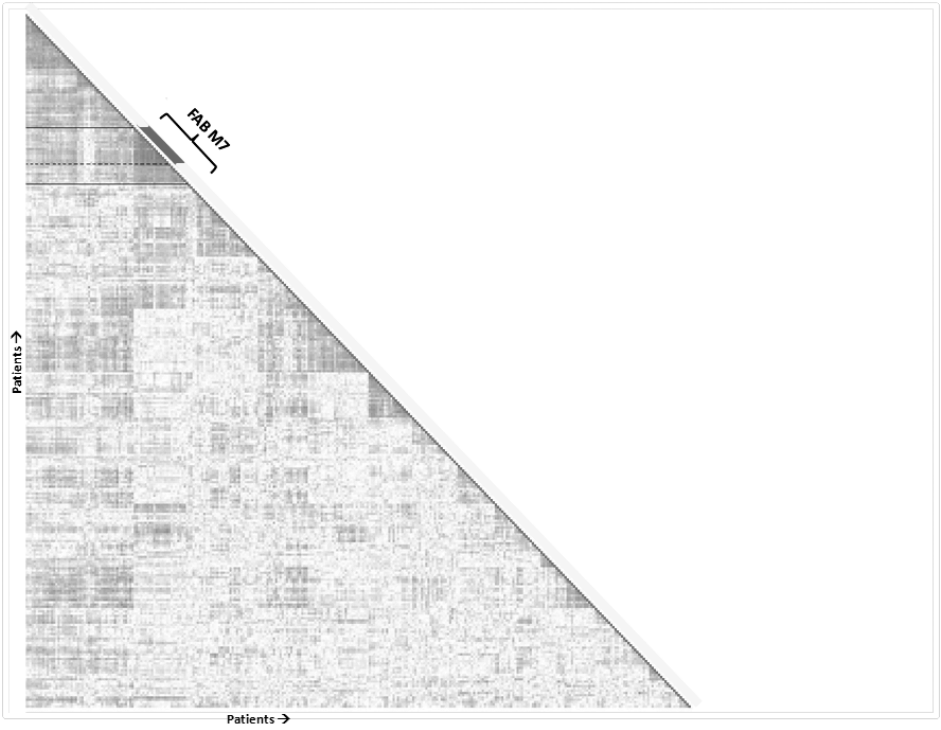

Figure 1
Unsupervised clustering of gene expression data revealed clustering of the ML-DS cases in the non-DS M7 pediatric leukemias.
Pair wise correlations between gene expression profiles of 297 *de novo* pediatric AML and 14 ML-DS samples, are displayed in a correlation plot. Colors of boxes represent the Pearson's correlation coefficient with a color gradient ranging from deep blue for a negative correlation, to vivid red for a positive correlation. Distinct clusters of samples, which can be recognized by the red blocks showing high correlation along the diagonal, are observed. The column to the right of the plot indicates the ML-DS patients (in blue), clustering all together within the non-DS AML FAB M7 leukemias. (see color figure on page 174)

days) with a median WBC of 104 x10^9/l (range 35- 410 x10^9/l), and 62% were male. The median age at diagnosis of the ML-DS patients was 1.9 years (range 0.9-2.5 years), the median WBC was 12 x10^9/l (range 3- 168 x10^9/l), and 39% of the patients were male. An overview of patient characteristics is given in Table 1.

Unsupervised clustering

Unsupervised cluster analysis of 296 children with *de novo* (non-DS) AML and 15 with ML-DS showed that all ML-DS cases formed a distinct cluster (see Figure 1). Of interest, this ML-DS cluster lies within the non-DS FAB M7 (acute megakaryocytic leukemia) pediatric AML cases. Other clusters within this AML cohort are not primarily formed by FAB-types, but e.g. by cytogenetic status.

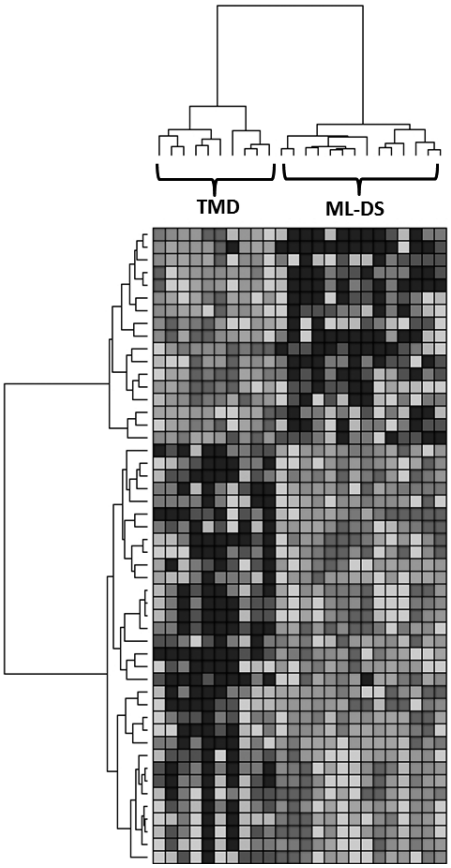

Figure 2
Hierarchical clustering based on the top 50 most discriminative genes for TMD and ML-DS patients.
Hierarchical clustering of 24 TMD and ML-DS cases (columns) based on their top 50 most discriminative genes (rows) (Table 2). (see color figure on page 175)

Gene signatures and overexpression of *MLLT3* and *ITGA4* in ML-DS compared to TMD

Microarray- based gene expression profiles of 10 TMD cases were compared with that of 14 ML-DS cases which identified a specific gene expression signature for both groups (Figure 2), based on the top 50 most discriminative genes (Table 2). In total, 1566 genes were differentially expressed (*P*- value <0.05) between these 2 disease entities. Interestingly, 3 of the top 100 most discriminative probe sets were probe sets for the (myeloid/lymphoid or mixed-lineage leukemia; translocated to, 3) *MLLT3*-gene, alias *AF9* (Table 2). The VSN-normalized mean average intensity of 3 of these probe sets (204917_s_at, 1569652_at, 204918_s_at) was significantly higher for ML-DS cases compared with TMD patients (*P* <0.0001) (Figure 3A). None of our ML-DS patients carried the *MLL-MLLT3* translocation, as screened by conventional cytogenetics and RT-PCR.

Table 2
Top 50 most discriminative probe sets for TMD and ML-DS
The genes above the line are downregulated in ML-DS (compared to TMD), the genes under the line are upregulated in ML-DS.

Probe_ID	Gene symbol	*p*-value
205463_s_at	PDGFA	3.97E-05
210493_s_at	MFAP3L	2.40E-04
207206_s_at	ALOX12	3.48E-04
231341_at	SLC35D3	5.10E-04
211743_s_at	PRG2	5.63E-04
1553960_at	SNX21	6.17E-04
205382_s_at	CFD	9.95E-04
204495_s_at	C15orf39	1.27E-03
209129_at	TRIP6	1.37E-03
219998_at	HSPC159	1.59E-03
229830_at	NA	1.64E-03
204214_s_at	RAB32	1.80E-03
201136_at	PLP2	1.91E-03
204135_at	FILIP1L	2.29E-03
210843_s_at	MFAP3L	2.34E-03
234199_at	—	2.45E-03
1559675_at		2.67E-03
227917_at	LOC157278	4.61E-05
204917_s_at	*MLLT3*	*1.48E-04*
205885_s_at	*ITGA4*	*1.86E-04*
213416_at	CERKL	2.94E-04
209616_s_at	CES1	4.02E-04

Table 2 (continued)

Probe_ID	Gene symbol	*p*-value
221021_s_at	CTNNBL1	4.56E-04
227623_at	NA	6.71E-04
207730_x_at	HDGFR2	7.25E-04
1567214_a_at	PNN	7.79E-04
218155_x_at	TSR1	8.33E-04
221253_s_at	TXNDC5	8.87E-04
224435_at	C10orf58	9.41E-04
212018_s_at	RSL1D1	1.05E-03
228155_at	C10orf58	1.10E-03
1552344_s_at	CNOT7	1.16E-03
202983_at	HLTF	1.21E-03
205884_at	*ITGA4*	*1.32E-03*
213194_at	ROBO1	1.43E-03
214707_x_at	ALMS1	1.48E-03
217713_x_at	NA	1.53E-03
212036_s_at	PNN	1.70E-03
226766_at	ROBO2	1.75E-03
222731_at	ZDHHC2	1.86E-03
203633_at	CPT1A	1.97E-03
217679_x_at	—	2.02E-03
222372_at	—	2.07E-03
227485_at	DDX26B	2.13E-03
215529_x_at	DIP2A	2.18E-03
214715_x_at	ZNF160	2.24E-03
233873_x_at	PAPD1	2.40E-03
214594_x_at	ATP8B1	2.51E-03
205786_s_at	ITGAM	2.56E-03
208082_x_at		2.61E-03

In addition, the integrin alpha 4 gene (*ITGA4)* was found twice in the top 50 most discriminative probesets and the mean expression of these probesets (205885_s_at, 205884_at) was also significantly higher in ML-DS that in TMD patients (P <0.0001) (Figure 3A).

These gene expression data were validated with RQ-PCR in 10 TMD and 16 ML-DS cases (Fig 3B and 4B). A high correlation between the gene expression profiling data and the RQ-PCR data was found for both genes (*MLLT3 Rs* (Spearman correlation coefficient) =0.7, p<0.001 (Fig S1); *ITGA4 Rs*= 0.9; p< 0.001 (Fig S2)). Mutational analyses of the whole *MLLT3* gene did not reveal mutations neither in TMD nor in ML-DS patients.

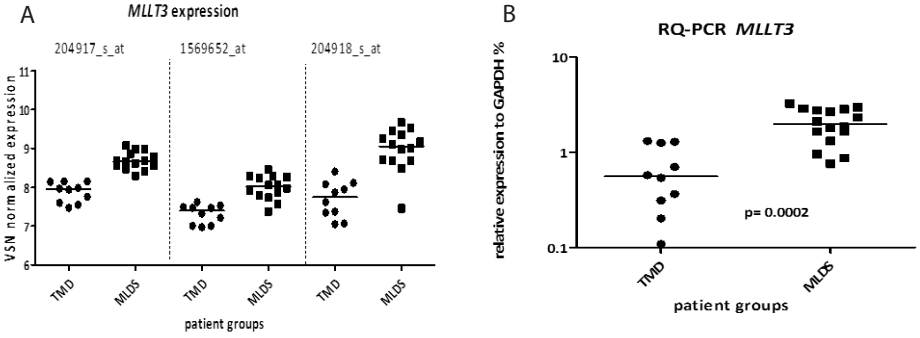

Figure 3A

Expression of *MLLT3* in TMD and ML-DS (GEP). ML-DS patients showed a higher average expression of 3 probe sets (204917_s_at, 1569652_at, 204918_s_at) than TMD-patients. The adjusted *P*- values are: p < 0.0001; p= 0.0002, and p=0.0002).

Figure 3B

Expression of *MLLT3* in TMD and ML-DS by RQ-PCR. RQ-PCR confirmed higher expression of *MLLT3* in ML-DS patients compared to TMD-patients; *P*- value < 0.0002

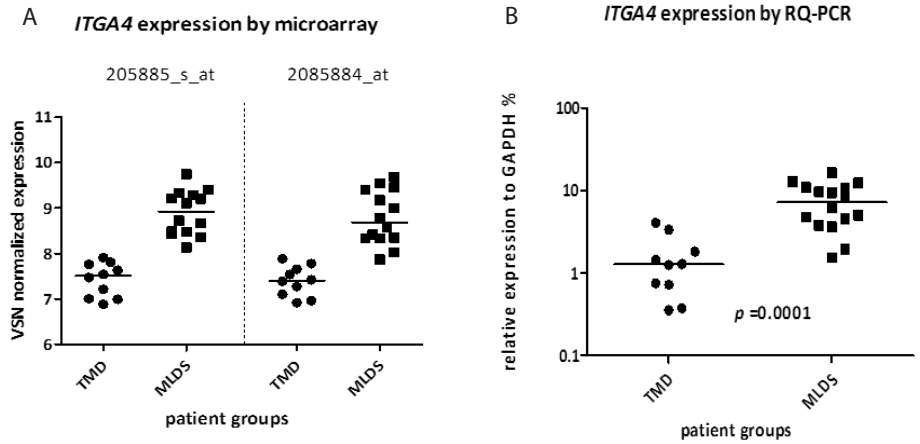

Figure 4A

Expression of *ITGA4* in TMD and ML-DS by micro-array.
ML-DS patients showed a higher *ITGA4* expression than TMD-patients. Adjusted *P*-values are 0.0002 (205885_s_at)
and 0.0013 (ps 205884_at).

Figure 4B

Expression of *ITGA4* in TMD and ML-DS by RQ-PCR.
RQ-PCR confirmed higher expression of *ITGA4* in ML-DS patients compared to TMD-patients; *P*-value < 0.001.

Knockdown of *MLLT3*

Unfortunately, no efficient knockdown of *ITGA4* could be established in the CMK ML-DS cell line, hence only functional data on *MLLT3* are presented.

To explore a possible role for *MLLT3* in cell proliferation, this gene was silenced by *MLLT3* siRNAs in the CMK cells. At several timepoints, the expression of *MLLT3* was measured (Fig 5A). We established an efficient knockdown by 75% compared to the non-target siRNAs mRNA levels. Western blot analyses also showed knockdown, of approximately 45% on protein level compared to the non-target control (Fig 5B).

The incorporation of BrdU did not differ between *MLLT3* siRNAs transfected cells and those transfected with non- target siRNAs, which may indicate that *MLLT3* does not influence cellular proliferation *in vitro* (Fig 5C).

To measure an anti-apoptotic effect of *MLLT3,* cell viability was tested by Annexin V/PI staining. *MLLT3* knockdown cells showed no differences in cell viability compared with cells transfected with non-target siRNAs (Fig 5D).

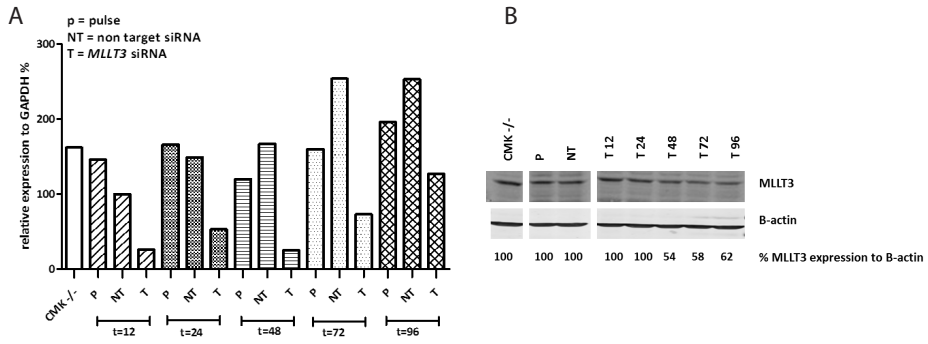

Figure 5A

Knockdown of *MLLT3* at mRNA level after transfection with siRNA directed against *MLLT3.*
Timepoints are shown in hours after electroporation (t=0).
CMK-/- = cell line without any pulse or siRNA at t=0; pulse = electroporation without any siRNA. NT = non target (control) siRNA. The expression of NT at t=12 is set at 100%.
This figure shows a knockdown of *MLLT3* at several timepoints after transfection.

Figure 5B

Knockdown of *MLLT3* at protein level at several timepoints after transfection with siRNA directed against *MLLT3*.
CMK-/- = cell line without any pulse or siRNA at t=0; pulse = electroporation without any siRNA. NT = non target (control) siRNA. The expression of NT is set at 100%.
This figure shows knockdown of MLLT3 at protein level at several timepoints (T12 =12 hours) after transfection.

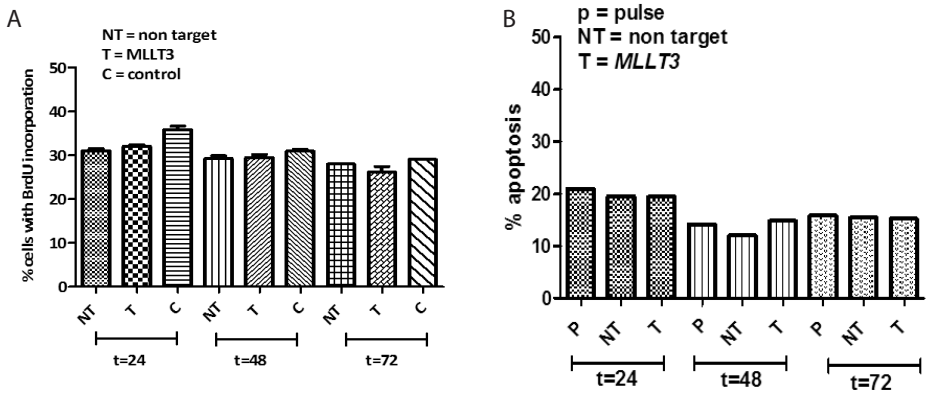

Figure 5C
Effect of knockdown of *MLLT3* on cell- proliferation.
NT = non target siRNA; T= *MLLT3* siRNA; C= control. This figure shows that knockdown of *MLLT3* (T) did not influence cell-proliferation. Compared to the non-target control (NT), the BrdU incorporation was identical.

Figure 5D
Effect of knockdown of *MLLT3* on apoptosis.
Pulse = electroporation without any siRNA. NT = non target (control) siRNA. T= *MLLT3* siRNA.
This figure shows that knockdown of *MLLT3* (T) did not influence apoptosis, compared to the non-target control (NT).

IGF1R

Given that *IGF1R* (representative for IGF signaling) has been reported constitutively overactive in ML-DS [14], we also studied the effect of *MLLT3* knockdown on *IGF1R* expression. *IGF1R* expression was measured in CMK cells at several timepoints after silencing *MLLT3*. Significantly lower expression levels of *IGF1R* were seen in the *MLLT3* silenced cells compared to the levels of the non-targeting control at early timepoints (t=12 and t=24 hrs; *P*= 0.03 and *P* = 0.03), Fig 6. However, *IGF1R* expression was similar in TMD and ML-DS patients (*P* = 0.8).

DISCUSSION

It is still not known which factors play a role in the progression from TMD to ML-DS. In the present study, we performed gene expression profiling in a cohort of TMD and ML-DS cases and identified specific gene expression signatures for both these disease entities.

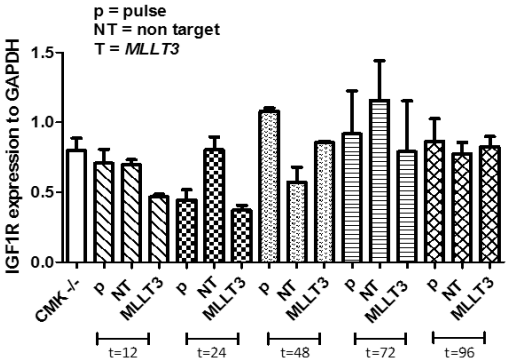

Figure 6

Expression levels of *IGF1R* after *MLLT3* knockdown.

CMK-/- = cell line without any pulse or siRNA at t=0; pulse = electroporation without any siRNA. NT = non target (control) siRNA. T= *MLLT3* siRNA. Timepoints are shown in hours after electroporation. This figure shows significantly decreased expression of IGF1R after knockdown of *MLLT3* at t= 12 and t =24 (P =0.03 and P =0.03).

MLLT3 (alias *AF9*) was one of the most significantly differently expressed genes between TMD and ML-DS. This gene is mainly known from *MLL*-rearranged leukemias and is, when fused to the *MLL*-gene *(*mixed lineage leukemia gene), able to initiate transformation from a committed progenitor into a leukemic stem cell [31]. Forced expression of *MLLT3* promotes the output of erythroid and megakaryocytic progenitors and it cooperates with *GATA1* in regulating the cell fate of the erythrocytes and megakaryocytes [32]. In a mouse model, no effect on hematopoietic stem cells was seen after inactivating the murine Af9 [33].

We hypothesized that *MLLT3* might play a role in the progression of TMD into ML-DS and that the differential gene expression of *MLLT3* might influence proliferation or apoptosis. However, *in vitro* studies by using siRNAs did not reveal any differences in proliferation and apoptosis between non-target transfected and *MLLT3*-silenced ML-DS cells. Neither did additional sequence analyses of the entire *MLLT3*-gene reveal any mutations in both TMD and ML-DS cases.

Klusmann *et al.* previously showed overactive IGF signaling in ML-DS [14]. The mitogenic and anti-apoptotic activity of IGF is mediated principally by the IGF1 receptor (*IGF1R*), which is known to play a role in AML [34]. We showed here that *IGF1R* expression levels were similar in TMD and ML-DS patients, which is in concordance with literature [14]. However, there was a significant decrease in *IGF1R* expression at the early timepoints after silencing *MLLT3* in the ML-DS cell line CMK. These data suggest that *MLLT3* functionally regulates *IGFR1*. Apart from *MLLT3,* GATA1s also plays a role in the regulation of *IGF1R*. *MLLT3* acts in a regulatory circuit including *GATA1* [32] and (full length) GATA1 intersects

with IGF signaling to restrict the proliferation of fetal megakaryocytic progenitors, whereas GATA1s contributes to leukemogenesis by perturbing this function of GATA1 [14].

*ITGA4 (*Integrin Alpha-4 or VLA 4-receptor Alpha4 subunit), located on chromosome 2, is part of the integrin family. Integrins are receptors involved in cell adhesion to extracellular matrix proteins, cell-surface ligands, and soluble ligands [35, 36]. Previous studies have described the importance of several integrins for AML, as they are involved in the binding of AML cells to bone marrow stroma and cellular migration, and may mediate anti-apoptotic signals and confer acquired drug resistance. Recently, it was shown that high expression of the VLA-4 integrin was associated with a reduced relapse rate in pediatric non-DS AML [37]. Conflicting with this observation, VLA-4 positive cells acquired resistance to anoikis (apoptosis induced by inadequate or inappropriate cell–matrix interactions) or drug-induced apoptosis in AML [38]. In our study, *ITGA4* was significantly higher expressed in ML-DS compared to TMD. This differential expression may be involved in the differences in homing of these diseases (fetal liver vs. bone marrow). Unfortunately, no efficient knockdown of this gene could be established in the ML-DS cell line, so we could not study the functional consequences of silencing this gene.

Our study is unique as we only used purified samples (>80% blasts) and the Affymetrix Human Genome U133 plus 2.0 microarrays containing >54000 probe sets to compare TMD and ML-DS. This is in contrast to Bourquin *et al.* who used gene expression profiling and showed distinct molecular phenotypes for ML-DS and non-DS AMKL, but TMD samples were not included [15]. Lightfoot *et al*. and McElwaine *et al* showed a distinct gene expression profile for TMD and ML-DS, but their platform contained less probesets (19.000) and unpurified samples [16, 39]. On the contrary, an important limitation of our study is that we did not have paired samples available, which in principle is the best model to study clonal evolution.

ML-DS is a unique disease entity and by using unsupervised clustering all ML-DS cases form a distinct cluster within the non-DS pediatric AML FAB M7 cases. Hence, ML-DS is a unique disease entity given its strong discriminative GEP profile.

In conclusion, we showed that TMD and ML-DS have distinct gene signatures, including high expression of *MLLT3* and *ITGA4* in ML-DS patients compared to TMD-cases. *MLLT3* knockdown did not influence proliferation or apoptosis in ML-DS, but resulted in a significant decrease of *IGF1R* expression at early timepoints, which may indicate that these genes interplay in the leukemogenesis of ML-DS. Further investigation of the role of *MLLT3* (in cooperation with *IGF1R*) in the progression of TMD into ML-DS is indicated.

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CHAPTER 7

SUMMARY GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Part of these data will be published in a review

SUMMARY

Children with Down Syndrome (DS) have a higher risk of developing leukemia [1-4]. This increased risk of leukemia includes both the risk for acute lymphoblastic leukemia (ALL), as well as acute myeloid leukemia (AML) [5]. Myeloid Leukemia of Down Syndrome (ML-DS) is a unique disease entity and its clinical and biological characteristics differ from AML in non-DS children, and therefore it is recognized as a separate disease entity in the WHO [6, 7]. Approximately 70% of all ML-DS cases have an excess of megakaryoblasts as in acute megakaryocytic leukemia (AMKL, FAB M7), the other cases are usually classified as undifferentiated leukemia (FAB M0). Furthermore, ML-DS is characterized by a low diagnostic white blood cell count, young age and a very low frequency of CNS involvement [8, 9].

Specific treatment guidelines for ML-DS patients have been developed, with reduced treatment intensity regimens. Recent results from several collaborative groups confirm the favorable outcome for this patient population with up to approximately 90% survival. DS newborns also have a unique predisposition to develop a clonal disease of varying severity during their first 3 months of life, i.e. transient myeloproliferative disorder (TMD), also referred to as transient leukemia. This disease resolves spontaneously in most cases, but a minority (about 20%) of these patients develop ML-DS in later life.

GATA1 is a transcription factor that regulates the differentiation of megakaryocytes and erythrocytes. Somatic mutations in the gene encoding for *GATA1*, localized on the X chromosome (Xp11.2), are pathognomonic for ML-DS [10, 11]. These mutations all lead to the truncated protein GATA1short (GATA1s), and mainly occur in exon 2, and are unique for every patient [12-14]. Both TMD and ML-DS are characterized by mutations in the *GATA1* gene, hence the evolution of TMD to ML-DS cannot be explained by mutations in GATA1. Although unique for every patient, some types of *GATA1*-mutations have been described to be significantly associated with lower white blood cell counts and a higher rate of progression to ML-DS after TMD [13], however this was not confirmed by others [15].

The progress that has been made in the field of cancer molecular genetics has provided us with techniques to further unravel the molecular genetic basis of TMD and ML-DS, which is warranted to get more insight in DS leukemogenesis, and may provide a model for leukemogenesis in general. In this thesis, we have used genome-wide techniques such as array-comparative genomic hybridization (A-CGH), gene expression profiling and direct sequencing to discover new molecular aberrations underlying TMD and/ or ML-DS.

In this thesis we assessed the genetic and molecular characteristics of ML-DS. In **chapter 2** we used genomic profiling (A-CGH) with the aim to detect new abnormalities involved in the pathogenesis of ML-DS, and to study the difference between TMD and ML-DS. Common regions of copy number alterations (CNAs) were found on chromo-

some 1q and on chromosome 11 in the ML-DS patients, mainly consisting of large (sub) chromosomal amplifications. Although we did not detect recurrent specific amplifications or deletions, TMD and ML-DS were characterized by a relatively high frequency of copy number alterations (CNAs) as compared to other pediatric AML subgroups (*MLL*-rearranged and cytogenetically normal AML), suggesting that ML-DS is genetically more unstable. As genomic instability can be the result of short and dysfunctional telomeres, we hypothesized that differences in telomere length were involved, but we could not detect any correlations between telomerase (*TERT*) mutations, telomere length and the number of CNAs in TMD and ML-DS. In conclusion, DS related leukemias are genetically more unstable, however, short telomeres do not seem to underlie this genetic instability.

In **chapter 3**, we performed mutational screening of the whole kinase and kinase-like domains of Janus kinases (JAK) 1-3 to determine the frequency in TMD and ML-DS cases. *JAK* belongs to a family of intracellular non-receptor protein tyrosine kinases that plays an important role in regulating the processes of cell proliferation, differentiation and apoptosis in response to growth factors. Activating mutations were previously described in TMD and ML-DS, however, most studies only focus on known mutations in *JAK* and hence may underestimate the mutational frequency. We also included a series of DS ALL patients as activating *JAK2*-mutations were also described in 20% of DS ALL patients. We did not detect a higher mutational frequency by screening the whole kinase and kinase-like domain than previously described, suggesting that there are clear mutational hotspots that can be screened for in clinical studies. Mutations were found in 2/15 ML-DS patients (*JAK1* and *JAK3*) and in 1/6 TMD-patient (*JAK3*). Of interest, a meta-analysis including our study and data from the literature confirmed the lack of prognostic significance for JAK2 mutations in DS ALL patients. In conclusion, we did not detect a higher mutational frequency by screening the whole kinase and kinase-like domain than previously described, suggesting that there are clear mutational hotspots that can be screened for in clinical studies.

Thereafter, aiming to further unravel the molecular signature of ML-DS, we studied the frequencies of (molecular) type-I and type-II aberrations in ML-DS in **chapter 4.** Type-I and type-II aberrations are involved in hyperproliferation and maturation arrest respectively, and their collaboration is required for the development of non-DS AML as postulated by Gilliland *et al* [16]. Recently, by using next generation sequencing technology, novel mutations have been discovered in AML genomes, including for instance *IDH1/2* and *DNTM3A* [17, 18], which have been shown to affect the epigenetic landscape of AML. This addition of epigenetic regulator mutations to the leukemogenic potential has recently to a revision of the Gilliland classification, adding a category of epigenetic regulator mutations [17]. The frequency of these type-I/II and epigenetic regulator aberrations had not yet been systematically investigated in ML-DS. Therefore, we screened ML-DS patients for recurrent gene aberrations in the *NPM1*, *CEPBPA*, *FLT3, KIT*, *PTPN11*,

N-RAS, *K-RAS*, *MLL*, *WT1*, *DNMT3A*, *IDH1*, and *IDH2* genes. Except for mutations in the *RAS*-gene (and SNPs in the *WT1* and *IDH1* genes), we did not detect any aberrations. When we looked into the expected frequency of the mutations in our ML-DS cohort compared to the non-DS AML pediatric AML patients (calculated from a binomial distribution), only the frequency of *WT1*, *FLT3-ITD*, and *N-RAS*- mutations appeared to be significantly less frequent. These results underscores the unique signature of ML-DS, and stresses the fact that further research is needed to unravel the cooperating molecular abnormalities involved in the leukemogenesis of this specific disease.

In **chapter 5** we aimed at detection of *novel* prognostic subgroups in ML-DS by conducting a large international study of clinical, cytogenetic and outcome data from ML-DS children, for which data were collected from 451 ML-DS cases of 13 collaborative study groups participating in the International-BFM Study Group. Karyotypes were available from 358 patients and were all centrally reviewed. Normal karyotype (NK) ML-DS, was shown to be an independent poor prognostic factor for overall survival, event-free survival, and relapse-free survival. It is conceivable that the NK-patients even may benefit from treatment intensification, as these patients have a higher incidence of relapse (21% vs. 9%) than treatment related mortality (6% vs. 7%), whereas treatment reduction may be feasible in ML-DS patients with cytogenetic abnormalities. Furthermore, high white blood cell counts (WBC $\geq 20 \times 10^9$/l) and older age (> 3years) at diagnosis were identified as independent predictors for poor event-free survival. Hence, this unique international collaborative study showed large differences in outcome within ML-DS patients and identified novel prognostic groups that predicted clinical outcome which may be used for stratification in future ML-DS treatment protocols.

In the last part of this thesis, **chapter 6**, we wanted to get more insight in the evolution of TMD into ML-DS. We approached this by using expression profiling (GEP) to study the differentially expressed genes involved in this evolution. The analyses showed a higher expression of the *MLLT3* (myeloid/lymphoid or mixed-lineage leukemia, translocated to, 3) and *ITGA4* (integrin alpha-4) gene*s* in ML-DS patients compared to (unpaired) TMD cases. This overexpression was conformed using RT-PCR. Functional experiments showed that in *vitro*, *MLLT3* knockdown did not influence proliferation or apoptosis in an ML-DS cell line (the CMK cell line). However, the knockdown did result in a significant decrease of *IGF1R* expression, which is known to be upregulated in (TMD and) ML-DS and contributing to leukemogenesis. Given this effect of *MLLT3* silencing on *IGF1R* levels, we hypothesize that the difference in expression might contribute to the development of ML-DS in contrast to the self-limiting character of most TMD cases. Furthermore, we performed unsupervised clustering of expression profiles of ML-DS cases showing that these cases represent a distinct cluster within non-DS pediatric AMKL cases. In conclusion, we found over-expression of *MLLT3* and *ITGA4* in ML-DS compared to TMD. *IGF1R*

was downregulated after *MLLT3* silencing, which may indicate that *MLLT3* plays a role in the regulation of *IGF1R* induced ML-DS leukemogenesis.

GENERAL DISCUSSION

The research presented in this thesis confirms that ML-DS is a unique disease entity and is not characterized by the spectrum of molecular genetic aberrations underlying sporadic pediatric AML. The development of AML at least requires two types of genetic events [16]. Type-I aberrations occur as mutations in hotspots of specific genes involved in signal transduction pathways, which lead to uncontrolled proliferation and/ or survival of leukemic cells. Type-II aberrations are often chromosomal rearrangements of transcription factors resulting in the translation of fusion proteins leading to impaired differentiation of the leukemic cells. This Gilliland hypothesis was further strengthened by observations from mouse models that one aberration is not sufficient to induce leukemia, but that cooperative events are needed to develop frank leukemia. For example, knock-in of *FLT3*/ITD leads to the development of a myeloproliferative disorder but lacks the maturation arrest typical for acute leukemia [19], whereas co-expression with inv(16)(p13q22) or t(15;17)(q22;q21) resulted in AML [20, 21]. Recent advances in technology have allowed the detection of many *novel* genetic and molecular abnormalities including cryptic translocations (such as *NUP98-NSD1*), and single gene mutations, occurring for instance in the *NPM1, CEBPA, WT1* and *MLL*-gene (*MLL*-PTD) which are predominantly found in patients with normal karyotype (NK)-AML[22, 23]. Newly discovered mutations, identified by whole genome sequencing, including mutations in the 'epigenetic regulator' genes encoding for *IDH1/ IDH2* and the DNA methyltransferase (*DNMT3A*) gene, are rare in sporadic pediatric AML [24, 25], but questioned whether the Gilliland model is still up to date. It appears to be more likely that they constitute an additional or several additional layers of (epi)genetic aberrations, which fits in the concept of a multistep pathogenesis model of AML and ML-DS.

Murine and *in vitro* studies showed that trisomy 21 itself is associated with expansion of erythro-megakaryocytic progenitors cells [26]. These findings were confirmed by Tunstall-Pedoe *et al*. who studied fetuses with DS at a gestational age of 15-37 weeks and found a marked increase in the megakaryocyte-erythrocyte progenitor pool in the fetal liver (without *GATA1*-mutations), as well as dysmegakaryopoiesis and dyserythropoiesis in the peripheral blood, concluding that trisomy 21 by itself induces a major change in normal multi-lineage myeloid hematopoiesis, and creates a window of opportunity for leukemic transformation [27, 28].

TMD and ML-DS are characterized by somatic mutations in *GATA1*, both in blood as well as in fetal liver samples [10, 29-35]. This transcription factor regulates the differentiation

of megakaryocytes and erythrocytes and is localized on the X-chromosome. Mutations that mainly occur in exon 2 lead to the truncated protein GATA1s and are unique for every patient [13, 14, 31]. It was shown that the short GATA1s isoform could not properly restrict megakaryocyte growth compared to full length GATA1 [36, 37], and results in excessive proliferation and, although to lesser extent, abnormal differentiation of an embryonic fetal megakaryocyte progenitor population [14]. In addition, a GATA1^{low} mutant mouse model showed that impaired GATA1 expression may contribute to the development of myelofibrosis [38]. So GATA1s might both induce maturation arrest and proliferation at the same time. This raises the question whether additional aberrations, next to GATA1s, are required for the development of ML-DS.

Cytogenetic analysis in ML-DS

Apart from the above mentioned molecular aberrations, the leukemic blasts from the majority of the patients with ML-DS (72%) show additional cytogenetic changes apart from the constitutional trisomy 21 [39]. A previous international-BFM study, performed by Forestier et al., showed that the most frequent gains involved chromosomes 8 (27%), 21 (23%), 11 (8.1%), and 19 (7.4%); whereas chromosomes X (3.2%; only females), 5 (1.5%), and 7 (2.2%) were commonly monosomic. The most frequent partial imbalances were duplication 1q (16%), deletion 7p (10%), and deletion 16q (7.4%) [39]. However, the potential impact of these cytogenetic abnormalities was not known and has not been well studied, mainly due to the small patient numbers in individual series [39-43]. In addition, no cytogenetic subgroups were identified within ML-DS until recently. Our international retrospective study showed large differences in outcome within ML-DS patients and identified normal karyotype (NK) as a poor prognostic factor for survival. The biological background of these NK ML-DS patients has to be investigated and thereby aberrations that possibly underly or explain this relatively poor prognosis may be discovered. As known from non-DS NK AML pediatric patients, mutations in *WT1*-gene and *FLT3/* ITD are associated with poor outcome, especially when they occur simultaneously [44]. In addition, *NUP98-NSD1* translocations confer a very poor prognosis [45]. We did not detect any of these mutations in 12 NK ML-DS patient samples, so other (epi)genetic aberrations may play a role in the poor prognosis of this NK ML-DS subgroup.

Besides the NK ML-DS patients, we identified a subgroup of trisomy 8 cases (with or without additional cytogenetic aberrations), and a subgroup with losses of chromosome 5/7 material. Non-DS pediatric AML with a trisomy 8 is classified as an intermediate risk group [46]. We showed significantly better outcome estimates for the trisomy 8 ML-DS patients in a direct comparison to non-DS AML trisomy 8 patients. Apparently, an additional copy of chromosome 8 has biologically different consequences in ML-DS compared to non-DS AML. Another prognostic cytogenetic abnormality in non-DS AML is monosomy 7 that is known to be a poor prognostic factor [47]. Outcome was signifi-

cantly worse in patients with a loss of the whole chromosome (monosomy 7) than in patients with a del(7q) in non-DS AML [46, 47]. In our ML-DS series, such differences were not observed, but numbers were small. Comparing ML-DS and non-DS AML patients revealed that ML-DS patients with monosomy 7 and/or del(7q) had a remarkably lower cumulative incidence of relapse, thus chromosome 7 aberrations do not seem to have the same implications in ML-DS as in non-DS pediatric AML. The discovery of the novel prognostic cytogenetic subgroups that predicted clinical outcome may be used for stratification in future treatment protocols.

Collaborative type-I and type-II aberrations in ML-DS and options for targeted therapy

The only recurrent mutations we identified in ML-DS were mutations in the RAS-pathway (9%). RAS-proteins were characterized as essential components of signalling networks controlling cellular proliferation, differentiation and survival. Oncogenic mutations of the *N-RAS* or *K-RAS* genes result in activation of these signalling pathways thereby leading to hyperproliferation and the development of malignancies [48]. RAS-pathway abnormalities are well known from non-DS pediatric AML patients [22], (e.g. represented the vast majority of type I aberrations in *MLL*-rearranged AML [49]) and underlie most of the juvenile myelomonocytic leukemia (JMML) cases [50]. In addition, *RAS* mutations were identified as an independent predictor for poor outcome in *MLL*-rearranged infant ALL [51].

It seems reasonable that *RAS*-mutations could be a target for future therapy options in a small subset of ML-DS cases, however developing therapeutic agents to directly block oncogenic RAS activity in *RAS*-mutant tumours has thus far been a challenging and unsuccessful endeavour [52]. Therefore, a great deal of effort is currently being applied to developing therapies that target effector pathways downstream of RAS, of which the Raf-MEK-ERK pathway (also known as the MAPK cascade) is well studied. MEK-inhibitors block this RAS-MAPK pathway, but often activate another pathway downstream of RAS –such as the PI3'-kinase-, and have shown little clinical benefit as single agents [52]. Currently, trials with several MEK-inhibitors (trametinib) are being designed and conducted in pediatric oncology.

Secondly, *JAK* mutations were detected in ML-DS and therefore may be an interesting therapeutic target. Janus kinases (*JAK*) belong to a family of intracellular non-receptor protein tyrosine kinases that transduce cytokine-mediated signals through the STAT family of transcription factors. *JAK* plays an important role in regulating the processes of cell proliferation, differentiation and apoptosis in response to growth factors. The *JAK2* V617F mutation is well known in myeloproliferative neoplasms (MPN) and results in the impaired ability of the pseudokinase domain to negatively regulate the kinase domain [53]. In MPN, several JAK-inhibitors are already used [54]. Remarkably, patients with

and without the *JAK2* V617F mutation may benefit to the same extent which is due to the fact that the current inhibitors do not differentially inhibit mutated and wildtype *JAK2,* as the location of the mutation is outside the ATP-binding pocket of the enzyme [55]. Treatment has shown to result in a decrease in organomegaly and improved quality of life in responding patients [54, 56].

Except for 1 ML-DS patient, carrying a missense *JAK1* mutation, mutations were only found in *JAK3* (in 14% of ML-DS patients) in our cohort. Of interest, these *JAK* mutated cases may be sensitive to JAK-inhibitors, which might be of benefit, since it is well-known that DS patients have an increased risk for chemotherapy related morbidity and mortality. A major side-effect of JAK2 inhibitors is myelosuppression which is due to suppression of wildtype JAK2 that is required for normal hematopoiesis [57], and which may render it difficult to combine JAK inhibitors with chemotherapy. In pediatric leukemias, the safety of the *JAK1-2* inhibitor Ruxolitinib is tested in an ongoing phase I study by the COG for patients with relapsed or refractory solid tumors, leukemia or myeloproliferative disease (Clinical Trial NCT01164163). The use of such an inhibitor may only be applicable in selected ML-DS cases. In addition, Roberts *et al*. described the importance of tyrosine kinase inhibitors in high-risk non-DS ALL characterized by rearrangements/translocations involving *JAK2* [58]. Such rearrangements have not yet been described nor studied in ML-DS.

Another therapeutical target by which JAK-STAT activation might be inhibited are the Pim-kinases; these are critical downstream effectors of among others JAK2 and other effector drivers of tumorigenesis. Recent investigations have established that the Pim kinases function as effective inhibitors of apoptosis and when overexpressed, produce resistance to the mTOR (mammalian target of rapamycin) inhibitor, rapamycin. Overexpression of the PIM-kinases has been reported in several other hematological and solid tumors (PIM1), myeloma, lymphoma, leukemia (PIM2) and adenocarcinomas (PIM3) [59]. As such, the Pim-kinases are a very attractive target for pharmacological inhibition in cancer therapy. Novel small molecule inhibitors of the human Pim-kinases have been designed and are currently undergoing preclinical evaluation [59].

Genome wide profiling

The introduction of advanced genome wide profiling may provide us with new aberrations that could play a role in ML-DS leukemogenesis. The application of array-CGH revealed a relatively high frequency of copy number alterations (CNAs) in ML-DS, suggestive of genomic instability, compared to non-DS pediatric AML cases. In addition, by using single-nucleotide-polymorphism (SNP) microarrays and candidate re-sequencing, it was shown that de novo (non-DS) pediatric AML is characterized by a low frequency of CNAs (average of 2.38 CNAs per case) [60], in contrast to pediatric acute lymphoblastic leukemia (average of 6 CNAs per case). The only exception to the low CNAs in AML was

AMKL, with the presence of a high number of CNAs but rare point mutations [61]. In accordance with our findings that TMD samples had fewer CNAs compared to ML-DS, TMD had fewer numbers of somatic mutations compared than ML-DS. While *GATA1* was the only recurrent mutational target in the TMD phase, mutations in e.g. *NRAS* and *TP53* genes were recurrently detected in ML-DS and AMKL samples [62]. Recently, Vogelstein *et al.* showed that in order to develop AML an average of 7.8 non-synonymous mutations were needed, which is lower than solid tumors [63], although these findings involved the AML M5 subtype and not AMKL (AML M7) nor ML-DS.

Genomic instability refers to an increased tendency of alterations in the genome during life cycle of cells and plays a role in tumorigenesis. During cell division, genomic instability is minimized by several major mechanisms: DNA replication, chromosome segregation in mitosis, repair of DNA damage, and coordinated cell cycle progression [64]. Telomeres cap the ends of chromosomes to protect them from damage, but shorten with each cell division, therefore genomic instability can be caused by short and dysfunctional telomeres [65]. We found that ML-DS had significantly more CNAs than other pediatric AML-subgroups (except for non-DS AMKL), suggesting that the DS related leukemia is genetically more unstable. We did not find evidence that the genomic instability in ML-DS was based on short telomeres and therefore one of the oher above mentioned mechanisms may be responsible for this phenomenon.

At the transcriptome level, gene expression profiling (GEP) has already proven its value in cancer research [66-68]. Altered gene expression levels may also play a role in DS leukemogenesis, and several overexpressed megakaryopoiesis-related genes encoded on chromosome 21 disturbing normal hematopoiesis have been described [69, 70]. For example, overexpression of *ERG* drives aberrant megakaryopoiesis. ERG also strongly cooperates with the GATA1s mutated protein, to immortalize megakaryocyte progenitors, suggesting that the additional copy of *ERG* in trisomy 21 may have a role in ML-DS [71]. In addition, miR125-b2, also located on chromosome 21, has been described as a potential oncomiR in TMD and ML-DS, as this miR functions in increasing the proliferation and self-renewal of megakaryocytic progenitors, by downregulating the direct targets *DICER1* and *ST18* [72]. Klusmann *et al.* also showed that insulin-like growth factor (IGF) signaling via the IGF1 receptor (*IGF1R*) is activated in ML-DS, and contributes to the survival and proliferation of ML-DS cell lines. This mitogenic pathway converges with mutated *GATA1s* in fetal megakayopoiesis and leukemogenesis [70]. We and others [73] identified specific gene expression signatures for both these disease entities, including higher expression of 2 genes, i.e. *MLLT3* and *ITGA4* in ML-DS patients compared to TMD-cases. Forced expression of *MLLT3* promotes the output of erythroid and megakaryocytic progenitors and it cooperates with *GATA1* in regulating the cell fate of the erythrocytes and megakaryocytes [74]. However, *in vitro* studies by using siRNAs did not reveal any differences in proliferation and apoptosis between non-target transfected and *MLLT3*-silenced ML-

DS cells, but resulted in a significant decrease of *IGF1R* expression at early timepoints, which may indicate that these genes interplay in the leukemogenesis of ML-DS. Further investigation of the role of *MLLT3* (in cooperation with *IGF1R*) in the progression of TMD into ML-DS is therefore indicated.

Unfortunately, the application of the above described advanced techniques did not reveal repetitive aberrations specifically for ML-DS leukemogenesis. The development of next-generation genome-wide sequencing and other techniques as methylation assays, profiling of histone modifications and miRNA expression profiling will probably help in unraveling the genetic landscape of the ML-DS genome and thereby may contribute to targeted therapies.

Treatment and prognosis of ML-DS

Before the 1980's, children with ML-DS were often treated with palliative care only. Ravindranath *et al.* (POG) and Lie *et al.* (NOPHO) were the first to report stable remissions in ML-DS patients who were treated with chemotherapy [75, 76]. Drug resistance profiles showed that the ML-DS blasts are relatively sensitive to various chemotherapeutic drugs *in vivo* and *in vitro* [77, 78], which enables dose-reduction. The increased chemosensitivity does not only apply to the leukemic cells but also to the healthy tissues and thereby contributes to increased treatment related morbidity and mortality [79]. Therefore, the application of dose-reduced treatment protocols combined with the development of more extensive supportive care resulted in excellent survival rates [8]. Currently, ML-DS has a favourable prognosis of 90% (3-yrs OS), according to the promising results in the AML-BFM-98 study. In the ML-DS 2006 treatment protocol ML-DS patients will receive the standard BFM chemotherapy regimen, with significant dose-reductions for cytarabine (28 g/m^2 versus 41 g/m^2–47 g/m^2 for non-DS patients) and anthracyclines (230 mg/m^2 versus 320 mg/m^2–450 mg/m^2 for non-DS patients).

Treatment of sporadic pediatric AML is stratified according to risk-group classification generally based on the presence of certain specific cytogenetic aberrations and the early response on treatment. Risk-group stratification is not being used in the current treatment ML-DS protocols, as there were no prognostic indicators available in ML-DS.

Children with ML-DS, who suffer from relapse, should be treated according to an individualized schedule, which takes the increased risk of toxicity and potential resistant disease into account. A recent study regarding refractory/ relapsed (R/R) ML-DS showed that clinical outcome for these patients is generally unfavorable (3-year OS of 25.9% +/- 8.5%) even in those receiving stem cell transplantation [80]. Stem cell transplantation should not be a standard practice, given the high risk of procedure related morbidity and mortality in DS children [81] (and ML-DS 2006 protocol), but may be considered. Only single successful cases have been reported [82].

Age has been recognized as a prognostic factor in ML-DS, with an inferior outcome in the limited number of children aged over 4 years [83]. In fact, it has been proposed that DS children who present over 4 years of age suffer from sporadic AML occurring in a Down syndrome child, rather than 'true' ML-DS [84]. Therefore screening for *GATA1* mutations is mandatory and cases lacking this mutation are therefore considered to resemble sporadic AML. Vice versa, some children with AM(K)L in fact have *GATA1*-mutated leukemia, suggestive of DS mosaicism [85].

Our retrospective international analysis revealed high white blood cell counts and age >3 years as predictors for clinical outcome. In addition, normal karyotype (NK) ML-DS was identified as an independent poor prognostic factor. As the incidence of relapse is higher than treatment related mortality in these latter cases, further therapy reduction is not indicated in this group; in fact treatment intensification may be needed. On the other hand, further treatment reduction may be feasible in ML-DS cases with aberrant karyotypes. Such treatment stratifications have to be validated in prospective clinical studies. In addition, detection of *GATA1*-mutations using patient-specific quantative PCR may be feasible as a marker for minimal residual disease (MRD) in the nearby future, but is not routinely used yet.

TMD screening and prevention of ML-DS

The true frequency of TMD is not known as this is only studied in selected populations [86]. As the course of TMD is uncomplicated and asymptomatic in the vast majority of cases, TMD is often undiagnosed prior to its spontaneous resolution. The suggested frequency of TMD in DS children is approximately 5% -10% [87], which is supported by the finding of *GATA1*-mutations in 590 Guthrie cards in 3.8% of newborn DS children [87]. However, mutations may have been missed due to subclonality, low numbers of cells or blasts, or extramedullary disease without circulating blasts. In addition, some DS foetuses may die in utero from causes other than TMD [88]. Published series are not population-based and thus the true incidence of TMD within DS patients cannot be directly ascertained [89, 90]. A population-based prospective study is currently ongoing within the Dutch Childhood Oncology Group, but results are not yet available. Other initiatives in the UK and in Germany are running in parallel.

There is a broad variety in the clinical presentation of TMD. Symptoms range from asymptomatic disease to massive organomegaly, liver failure and/or respiratory failure, which may lead to death in approximately 10-20% of the cases [91, 92]. Although TMD resolves in the majority of DS infants, about 20% of the patients subsequently develop ML-DS, generally between 1 and 4 years of age [90, 91, 93]. ML-DS develops either by overt progression or after apparent remission of TMD. Acquired chromosomal abnormalities appeared to be a risk factor for developing ML-DS in children with TMD [94], however,

the crucial genetic events controlling the progression/ evolution from TMD into ML-DS remain uncertain and are object of further study.

A relevant question is whether the development of ML-DS can be inhibited or prevented by treating children for (asymptomatic) TMD. To date, published series described similar rates of ML-DS development in treated and untreated symptomatic TMD patients [91, 92]. In addition, ML-DS-patients with a history of transient TMD had a significantly better outcome than ML-DS patients without documented TMD [91]. However, as there is currently no standard screening for TMD in DS newborns, it is not known whether all ML-DS patients had a history of TMD. Currently, an AML-BFM-Study Group chemoprevention trial is ongoing for (a)symptomatic TMD-patients in the Netherlands and Germany. This study is based on the hypothesis that eradication of the GATA1s clone may prevent the development of ML-DS eventually. DS newborns will be screened for TMD, and if TMD is detected, those who have symptoms or those who have persistence of blasts/ high minimal residual disease (MRD) by using GATA1s RQ-PCR or flowcytometry, will be treated with low-dose cytarabine. This is an ongoing prospective study and not standard of care, and we have to await the results (Dutch Trial Register TC1667).

The multi-step progression to ML-DS provides insight into the steps by which normal hematopoietic stem cells or progenitors are transformed into leukemic cells. Moreover this is an excellent disease model to understand cell type–specific signalling pathways and their intersection with oncogenes during malignant transformation. In pediatric leukemias, it had been described that the majority of chromosomal translocations arise in utero, however these translocations and pre-leukemic clones arise at a substantially higher frequency, reflecting the requirement for complementary and secondary genetic events that occur postnatally [19]. Therefore, TMD and the progression into ML-DS may serve as a model for leukemogenesis in general.

CONCLUSION AND FUTURE PERSPECTIVES

The research presented in this thesis confirms that ML-DS is a unique disease entity, that in most cases is characterized by different genetic and molecular alterations when compared to AML in non-DS children, in addition to the pathognomonic *GATA1*-mutations.

Currently, ML-DS patients have a 5-year overall survival of ~ 80-90%. In the last decade, improvement of survival was mainly reached by the reduction of chemotherapy and improved supportive care. This reduction was possible since ML-DS is a chemo-sensitive disease, as demonstrated by various groups including our own, using total-cell kill assays. Moreover, therapy reduction was needed as ML-DS patients suffered from higher treatment related morbidity and mortality when treated at full dose, causing especially higher rates of infections, bone marrow suppression and mucositis.

However, to date, no risk–adapted therapy has been applied in ML-DS treatment protocols, as no predictive biomarkers were identified. Our retrospective study identified *novel* subgroups that predict clinical outcome, which may be used for treatment stratification in the future. NK ML-DS is a poor prognostic subtype in which the events are dominated rather by induction failure and a high incidence of relapse than by treatment related mortality, and therefore treatment intensification may be necessary to enhance outcome. Especially intensifying the use of nucleoside analogs is an option as they are usually better tolerated than for instance anthracyclines in terms of mucositis and bone marrow suppression. In contrast, ML-DS cases with additional aberrations might benefit from further therapy reduction, for instance by replacing an anthracycline containing block by high-dose cytarabine with the aim to reduce long-term risk of cardiac toxicity. Current efforts for risk-group stratification should also focus on the prognostic significance of minimal residual disease to predict response to therapy, using patient-specific quantitative PCR to follow the levels of mutated *GATA1*, following the relevance of MRD for risk-group stratification in leukemia in general.

Alternatively, targeted therapy options may be explored. We identified that some ML-DS cases are characterized by genetic aberrations in the *JAK* and/or *RAS* genes, and these pathways may be targeted with kinase-inhibitors, although there are currently no studies showing a benefit of this, for instance in non-DS AML. In addition, the percentage of ML-DS patients with such aberrations is limited, which makes it difficult to perform clinical studies adequately powered to analyse this in ML-DS.

The presence of a large number of cases with chromosome 7 abnormalities in our ML-DS cohort and the specific prognostic relevance of chromosome 7 abnormalities in non-DS AML, focused our attention on this group separately. In ML-DS, chromosome 7 aberrations however do not seem to predict poor survival, which is intriguing and not understood to date. Apparently, these aberrations lack prognostic significance in the context of constitutional trisomy 21, in contrast to non-DS AML.

Our genome wide profiling studies showed that ML-DS is genetically more unstable than TMD and most subgroups of non-DS AML. In addition, distinct gene expression signatures for TMD and ML-DS were shown, including high expression of *MLLT3* in ML-DS. This gene may interplay with *IGFR1* in ML-DS leukemogenesis.

There are several relevant biological questions to answer in the near future. First of all, the genetic background of ML-DS with a normal karyotype is not known, and elucidating the driving genetic abnormalities in these cases, for instance using the novel next-generation sequencing approaches is important, also in the light of their relatively poor outcome as discussed above. This may also reveal novel treatment targets. The second relevant question which is not resolved to date is which abnormalities drive clonal evolution from TMD to ML-DS, which only occurs in ~20% of the TMD cases. This is currently being studied in a longitudinal project using next generation sequencing

of paired samples (germ-line, TMD and ML-DS). A better understanding of the genetic events that drive the progression to ML-DS may also be of value in selecting patients that may benefit from treatment at the stage of TMD trying to prevent later progression to ML-DS. Such a study is currently ongoing using minimal residual disease *GATA1* levels 2-3 months postnatally in patients with TMD, but results are not yet available.

Finally, it is essential to screen newborn children with DS for the occurrence of TMD, as TMD may be associated with serious complications resulting in death in ~20% of children with TMD, although this may be an overestimation due to selection-bias. Diagnosing patients with TMD will also identify the children with DS at risk for developing ML-DS. Treatment of TMD with low-dose cytarabine can prevent TMD-related deaths, and requires the development of standardized TMD protocols. This, like all other research projects in DS leukemia, requires international collaboration, such as in the AML committee of the International BFM Study Group, given the rarity of these diseases.

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CHAPTER 8

NEDERLANDSE SAMENVATTING

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Kinderen met syndroom van Down (DS) hebben een verhoogde kans op het ontwikkelen van leukemie (bloedkanker). Dit betreft zowel acute lymfatische leukemie (ALL) als acute myeloïde leukemie (AML). Bij acute leukemie vermenigvuldigen onrijpe witte bloedcellen zich razendsnel en stapelen zich op in het beenmerg (de bloedfabriek), waardoor de productie van normale witte bloedcellen, rode bloedcellen en bloedplaatjes verstoord raakt. AML is een heterogene ziekte, omdat de leukemie kan ontstaan uit verschillende types voorlopercellen: namelijk voor rode bloedcellen, bloedplaatjes en bepaalde soorten witte bloedcellen, die allen tezamen myeloïde voorlopercellen worden genoemd. AML bij kinderen met DS wordt ML-DS (Myeloid Leukemia of Down Syndrome) genoemd. Dit is een unieke ziekte met andere klinische en biologische eigenschappen dan AML bij kinderen zonder DS.

Patiënten met ML-DS zijn jonger en de leukemie wordt gekenmerkt door een laag aantal witte bloedcellen bij het stellen van de diagnose. Bij de meerderheid van de ML-DS patiënten is er sprake van acute megakaroyblastische leukemie (FAB M7). Dit is een leukemie die ontstaat uit de voorlopercellen van de bloedplaatjes. Kinderen met ML-DS hebben een betere overleving dan kinderen met AML zonder DS (non-DS AML), omdat ze gevoeliger zijn voor chemotherapie. Daarentegen hebben ze meer last van de bijwerkingen van de behandeling. Daarom zijn er tegenwoordig speciale protocollen ontwikkeld voor ML-DS patiënten. Deze bevatten het standaard chemotherapieschema voor AML, maar met een lagere dosis van bepaalde cytostatica. Recente resultaten laten zien dat ongeveer 90% van de kinderen met ML-DS hiermee kan worden genezen.

Ongeveer 10% van alle pasgeborenen met DS ontwikkelt een tijdelijke ophoping van leukemiecellen in het bloed die wordt aangeduid als transient myeloproliferative disorder (TMD). Deze leukemiecellen ontstaan uit myeloïde voorlopercellen in de foetale lever, waar de foetale hematopoïese (bloedaanmaak) plaatsvindt. De meeste kinderen met TMD hebben geen klachten waardoor de diagnose makkelijk gemist kan worden. Als er klachten zijn (symptomatische TMD) wordt de diagnose meestal in de eerste 2 levensweken gesteld. Het overgrote deel van de kinderen met TMD herstelt spontaan, echter een klein deel van de TMD patiënten behoeft behandeling of overlijdt zelfs aan de ziekte. Een deel van de DS kinderen met TMD (~20%) ontwikkelt vervolgens ML-DS. Het is niet bekend of ML-DS ook voorkomt zonder voorafgaande TMD, want er wordt niet standaard gescreend op het voorkomen van TMD bij pasgeborenen met DS.

Zowel de TMD als de ML-DS leukemiecellen worden gekarakteriseerd door een verandering (mutatie) in het *GATA1*-gen (een gen is een stukje DNA dat codeert voor aminozuur dat uiteindelijk tot eiwitvorming leidt). Dit gen is gelokaliseerd op het X-chromosoom en speelt een rol in de uitrijping van de voorlopercellen van de bloedplaatjes en rode bloedcellen. Mutaties in het *GATA1*-gen zijn kenmerkend voor TMD en ML-DS en zijn

uniek voor iedere patiënt. Omdat deze mutaties zowel bij TMD als bij ML-DS voorkomen, moet er tenminste nog één extra factor verantwoordelijk zijn voor de progressie van TMD naar ML-DS. Deze factor(en) zijn tot op heden nog niet opgehelderd.

In de afgelopen jaren zijn er grote technische vooruitgangen geboekt op het gebied van de moleculaire genetica, wat de mogelijkheid biedt om de genetische veranderingen in het DNA van leukemiecellen steeds verder te ontrafelen. In dit proefschrift wordt ingezoomd op de genetische achtergrond van leukemiecellen van TMD en ML-DS. In het eerste deel van dit proefschrift hebben we de frequentie en karakteristieken van DNA veranderingen bij TMD en ML-DS bestudeerd. Vervolgens hebben we gekeken naar prognostische groepen binnen ML-DS. Tenslotte hebben we de verschillen tussen TMD en ML-DS onderzocht om te kijken welke factoren een rol zouden kunnen spelen bij de progressie van de ziekte.

In **hoofdstuk 2** pasten we de techniek van genoombrede array-comparative genomic hybridization (A-CGH) toe op materiaal van 22 ML-DS patiënten. Met deze techniek kunnen veranderingen in het aantal kopieën van alle chromosomen in één leukemie sample gedetecteerd worden: copy number veranderingen (CNAs). Deze CNAs kunnen een rol spelen in het ontstaan van leukemie. ML-DS patiënten hebben significant meer CNAs dan groepen van AML patiënten zonder DS (behalve de non-DS acute megakaryoblastische leukemie-subgroep). Dit suggereert dat Down syndroom leukemie genetisch meer instabiel is. Ook is er een toename te zien van CNAs in ML-DS vergeleken met TMD. Omdat deze genomische instabiliteit het resultaat kan zijn van korte telomeren (het uiteinde van een chromosoom), hebben we ons cohort tevens gescreend op mutaties in het telomerase reverse transcriptase (*TERT*) gen en hebben we gekeken naar telomeer lengte. Beide bleken niet gecorreleerd te zijn met de genomische instabiliteit in TMD en ML-DS. Derhalve kon deze instabiliteit niet verklaard worden door korte telomeren.

In **hoofdstuk 3** onderzochten we de frequenties van mutaties in de *Janus Kinase* genen (*JAK1-3*) in TMD (n= 6) en ML-DS (n= 15) patiënten. *JAK* genen spelen een rol in de regulatie van proliferatie (groei), differentiatie en apoptose (celdood) in de cel. Activerende *JAK* mutaties zijn eerder beschreven bij TMD en ML-DS. In ons cohort zie we dat *JAK* mutaties relatief zeldzaam zijn bij TMD en ML-DS; één ML-DS patiënt had een *JAK1* mutatie en *JAK3* mutaties werden gevonden in 1 TMD en 1 ML-DS patiënt. Deze laatste patiënten zijn in continue complete remissie (follow up 1.4 en 1.9 jaar), hoewel ook beschreven is dat *JAK3* mutaties geassocieerd zijn met een meer agressieve vorm van ML-DS. Tevens hebben we een serie DS ALL patiënten geïncludeerd. *JAK2* mutaties kwamen relatief vaak voor bij deze patiënten (17%), met name in een mutational hotspot binnen dit gen (*JAK2* R683). Geen van onze DS ALL patiënten met een JAK2 mutatie kreeg een recidief. Een meta-analyse toonde geen prognostische waarde voor DS ALL patiënten met een *JAK2* mutatie.

In **hoofdstuk 4** bestudeerden we de bekende genetische en moleculaire afwijkingen (die tot op heden beschreven zijn in non-DS kinder-AML) bij ML-DS patiënten, aan de hand van de indeling in de zogenaamde type-I en type-II afwijkingen. Type-I afwijkingen zijn de genetische afwijkingen in een leukemiecel die leiden tot de ongeremde groei (proliferatie). Type-II afwijkingen veroorzaken een stop in de uitrijping (maturatie stop) van de leukemiecel. Deze twee typen afwijkingen zijn minimaal nodig voor het ontstaan van leukemie uit een normale cel. We hebben ML-DS patiënten gescreend op afwijkingen in de *NPM1*, *CEPBPA*, *FLT3, KIT*, *PTPN11*, *N-RAS*, *K-RAS*, *MLL*, *WT1*, *DNMT3A*, *IDH1*, en *IDH2* genen. Afgezien van mutaties in het *RAS*-gen hebben we geen enkele andere afwijking gevonden bij de ML-DS patiënten. Wanneer we kijken naar de verwachte frequenties van de afwijkingen in ons ML-DS cohort vergeleken met de frequentie bij non-DS kinder-AML patiënten, bleek de frequentie van *WT1*, *FLT3-ITD*, en *N-RAS* mutaties significant lager. Het gebrek aan significante resultaten voor de overige afwijkingen kan bepaald zijn door de lage frequenties van deze afwijkingen in non-DS kinder-AML. Deze studie onderstreept het unieke karakter van ML-DS.

Vervolgens hebben we in een groot internationaal samenwerkingsverband (**hoofdstuk 5)** onderzocht of er bepaalde (cytogenetische) subgroepen zijn binnen ML-DS met prognostische waarde. De meeste ML-DS samples worden gekarakteriseerd door bijkomende afwijkingen in het karyotype naast de constitutionele trisomie 21. Wij bestudeerden de cytogenetische afwijkingen van ML-DS patiënten en relateerden deze aan overleving, waarbij we met name keken naar het voorkomen van een recidief van de leukemie (cumulatieve incidentie van recidief: CIR). De groep patiënten met een hoge CIR bestonden voornamelijk uit casus met een normaal karyotype (NK). Deze patiënten hadden ook een significant slechtere overleving. Omdat deze NK ML-DS patiënten een hogere incidentie hebben van recidief leukemie dan van behandeling- gerelateerde mortaliteit, is verdere reductie van de therapie niet geïndiceerd; wellicht is zelfs intensivering van behandeling noodzakelijk. Bij ML-DS patiënten met cytogenetische afwijkingen is reductie van behandeling is mogelijk wel toepasbaar. Tevens zijn een hoog witte bloedcel- getal ten tijde van de diagnose en leeftijd ouder dan 3 jaar ook gevonden als voorspellers voor slechtere event- vrije overleving. Deze unieke internationale studie toonde grote verschillen in overleving binnen ML-DS patiënten en heeft nieuwe prognostische subgroepen geïdentificeerd die overleving voorspellen en mogelijk als stratificatie gebruikt kunnen worden in toekomstige ML-DS behandelprotocollen.

Tenslotte identificeerden we specifieke gen expressieprofielen (**hoofdstuk 6**) voor TMD en ML-DS. Analyse van gen expressieprofielen toonde dat ML-DS casussen een eigen cluster vormden binnen non- DS AML. Tevens vonden we dat het *MLLT3*-gen en *ITGA4*-gen onderscheidend waren voor ML-DS, daar de mediane expressie (bevestigd door RQ-PCR) van beide genen significant hoger was in ML-DS dan in TMD- patiënten. Additionele analyses voor *MLLT3* toonde geen mutaties welke verantwoordelijk zouden

kunnen zijn voor het verschil in expressie. Het 'uitzetten' van het *MLLT3*-gen had geen invloed op proliferatie (celgroei) of apoptose (celdood) in ML-DS, maar resulteerde wel in een significante afname van *IGF1R* expressie, wat kan betekenen dat deze genen samenwerken in de ontwikkeling van ML-DS. Daar is in de toekomst verder onderzoek naar nodig.

Concluderend hebben wij in dit proefschrift laten zien dat ML-DS een aparte ziekte-entiteit is gekarakteriseerd wordt door specifieke genetische afwijkingen, anders dan AML bij kinderen zonder DS. Tevens blijkt ML-DS genetisch meer instabiel te zijn dan (de meeste) non-DS kinder-AML subgroepen. Wij hebben prognostische subgroepen binnen ML-DS geïdentificeerd. Deze bevindingen zouden dan ook gebruikt kunnen worden om de ML-DS patiënten in te delen in zogenaamde risicogroepen, zodat de therapie toegespitst kan worden op de verschillende subgroepen. Prospectieve studies zijn noodzakelijk om te identificeren in welke mate en voor welke specifieke subgroepen de therapie verder kan worden aangepast. Wij hebben beschreven dat TMD en ML-DS verschillende gen expressie profielen hebben. Echter, de (epi)genetische afwijkingen verantwoordelijk voor de progressie van TMD naar ML-DS zijn tot op heden onbekend. Het is waarschijnlijk dat de toepassing van geavanceerde moleculaire technieken, zoals whole genome sequencing ons inzicht in de genetische achtergrond van TMD en ML-DS in de nabije toekomst zal doen toenemen. Deze kennis geeft niet alleen meer inzicht in het ontstaan van leukemie bij Down syndroom maar kan ook als model dienen voor leukemogenese in het algemeen.

ABOUT THE AUTHOR

CURRICULUM VITAE

Marjolein Blink werd op 18 februari 1980 geboren te Groningen. Na het behalen van haar Gymnasium diploma aan het Dr. Nassau College te Assen in 1998, startte zij met de studie Geneeskunde aan de Rijksuniversiteit Groningen. Naast haar studie volgde zij ook vakken aan de faculteiten Economie, Rechten en Communicatiewetenschappen. Zij sloot haar doctoraalfase van Geneeskunde af met een wetenschappelijke stage op de afdeling Kindernefrologie van het AMC te Amsterdam. In de klinische fase van haar opleiding koos ze voor het keuze-coschap Kindergeneeskunde en behaalde haar artsexamen in 2005. Hierna werkte zij ruim twee jaar met veel plezier als arts-assistent op de afdeling Kindergeneeskunde van achtereenvolgens het Rijnland ziekenhuis te Leiderdorp, het St. Antonius ziekenhuis te Nieuwegein en het ErasmusMC- Sophia Kinderziekenhuis te Rotterdam. Om haar wetenschappelijke horizon te verbreden startte zij in oktober 2007 als arts-onderzoeker op het promotieonderzoek 'Clinical relevance of genetic alterations in Myeloid Leukemia of Down Syndrome' op de afdeling Kinderoncologie/-hematologie van het ErasmusMC- Sophia. Het resultaat van dit onderzoek is beschreven in dit proefschrift. Marjolein is tevens werkzaam geweest als datamanager voor de Stichting Kinderoncologie Nederland (SKION, 2008-2009). In januari 2012 begon zij als arts-assistent Kindergeneeskunde in het VU Medisch Centrum te Amsterdam. Op 1 juni is zij aldaar gestart met de opleiding tot kinderarts (opleider Prof. Dr. R.J.B.J. Gemke). Thans is zij werkzaam in het Onze Lieve Vrouwe Gasthuis te Amsterdam. Ze heeft een relatie met Wouter Knaack.

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PHD PORTFOLIO

Summary of PhD training and research

Name PhD student: M. Blink
Erasmus MC Department: Pediatric Oncology
Research School: Molecular Medicine (MM)
PhD-period: 1 oktober 2007- 1 oktober 2011
Promotor: Prof. Dr. R. Pieters
Co-promotors: Dr. C.M. Zwaan, Dr. M.M. van den Heuvel-Eibrink

PhD training	Year
General courses	
Biomedical English Writing and Communication	2009
Classical methods for Data Analysis	2007
Specific courses	
Micro-array Data Analysis using R & Bioconductor Intensive Course	2009
Applied Bioinformatics (MM)	2008
Basic and Translational Oncology (MM)	2007
Biomedical Research Techniques	2007
Seminars and workshops	
Annual Molecular Medicine Day, ErasmusMC	2008-2011
Annual Pediatric Oncology Symposium, ErasmusMC	2008-2011
Annual PhD Day, ErasmusMC	2008-2010
Annual Pediatric Research Day, ErasmusMC	2008-2011
Presentations	
See 'seminars and workshops' and '(inter)national conferences'	2008-2011
7 oral presentations at the weekly Pediatric Research Meetings and Pediatric Oncology Research Meetings	

PhD training	Year
(Inter)national conferences	
IKR conference, Rotterdam (oral presentation)	2011
7th Bi-Annual I-BFM Leukemia symposium, Antalya, Turkey (oral presentation)	2010
Symposium Hematomorfologie Rotterdam (oral presentation)	2010
Down syndroom symposium, Breda (oral presentation)	2010
51st ASH Annual Meeting, New Orleans, USA (poster presentation 2x)	2009
19th Annual Meeting of the I-BFM Study Group, Glasgow, UK (oral presentation)	2008
50th ASH Annual Meeting, San Francisco, USA	2009
51st ASH Annual Meeting, New Orleans, USA (poster presentation 2x)	2008
Werkgroep Kindertumoren Symposium, Amsterdam (oral presentation)	2008
SKION dagen, Utrecht (oral presentation)	2008

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Marjolein

APPENDICES

SUPPLEMENTARY DATA

CHAPTER 3

Table S1

Clinical and genetic characteristics of the included TMD and ML-DS patients

ID	Disease	Sex	Age	WBC	Karyotype	*JAK*	*GATA 1* mutation
1	TMD	Male	1 day	64	NA	JAK3 A573V	itd 105 bpt
2	TMD	Female	2 days	193	47,XX,+21c	WT	del 2 bp
3	TMD	Male	1 day	35	NA	WT	ins 14 bp
4	TMD	Female	2 days	173	NA	WT	del 1 bp, ins 2 bp
5	TMD	Male	6 days	NA	NA	WT	del 2 bp
6	TMD	Male	1 day	410	NA	WT	ins 14 bp
7	ML-DS	Female	0,7	4	NA	JAK3 A573V	del 4 bp
8	ML-DS	Female	2,2	7	45-46,XX,der(1)t(1;6)(q31;q?),ins(4;1)(q12;q25q44),-6,-7,der(7)t(6;7)(p21;p22),der(7)t(7;8)(q2?2;q2?3),der(11)t(7;11)(p14;p15),+21c,der(22)t(1;22)(q25;p11)[cp13]/47,XX,+21c[28]	JAK 1 D625R	ins 7 bp
9	ML-DS	Male	1,3	6	47,XY,+21c 2/47,idem,t(4;15),(q?21;q?21),del(7)(q?31q?33)	WT	ins 16 bp
10	ML-DS	Male	2,0	26	NA	WT	del 2 bp
11	ML-DS	Female	2,2	9	NA	WT	del 2 bp
12	ML-DS	Female	2,0	168	NA	WT	pointmutation
13	ML-DS	Female	2,0	6	NA	WT	ins 1 bp
14	ML-DS	Male	2,3	12	NA	WT	ins 14 bp
15	ML-DS	Female	0,9	19	NA	WT	del 5 bp
16	ML-DS	Male	1,8	7	47,XY,+21c [3]	WT	ins 4 bp
17	ML-DS	Male	1,4	6	48,X,ins(Y;5)(q11;?),der(3)t(3;6)(q2?8;?) or ins(3;6)(q2?8;?),-5,del(6)(q1?4q2?4),+21c,+21,+mar [24]	WT	del 3 bp, ins 5 bp
18	ML-DS	Female	2,3	49	47,XX,der(9)inv(9)(p24;q2?1)del(9)(q2q3) [14]	WT	del 3 bp
18	ML-DS	Female	1,9	40	47,XX,der(1)t(1;1)(p36;q21),t(5;6)(p15;p23),+21c [23]	WT	del 6 bp
20	ML-DS	Female	2,4	3	47,XX,r(7)(p22q22).ish r(7)(WCP7+,D7Z1+,D7S486-,164D18-,3K23-),+21c	WT	pointmutation

WBC= white blood cell count (*10^9/l); WT = wildtype; NA = not available.

Age in years (except for TMD). Karyotype nomenclature according to ISCN 1995

Table S2

Clinical and genetic characteristics of the included DS ALL patients

ID	IPT	Sex	Age	WBC	Karyotype	*JAK*
21	C-ALL	Male	3,1	7	48,XY,+X,+21c[19]	JAK 2 R683 ins GGCCCCATC
22	C-ALL	Male	2,0	112	47,XY,+21c	JAK 3 R1092C
23	C-ALL	Female	2,7	2	50,XX,+X,+4,+17,+21c[5]/47,XX,21c[5]	JAK 2 R683
24	C-ALL	Male	2,2	18	47,XY,+21c[20]	JAK 2 R683
25	PRE-B ALL	Male	3,8	3	47,XY,+21c[22]	JAK 2 R683
26	C-ALL	Female	3,4	17	47,XX,+21c[20]	JAK 1 V651M
27	C-ALL	Male	3,3	4	47,XY,+21c	JAK 2 R683
28	PRE-B ALL	Female	6,2	5	46,XX,der(14;21)(q10;q10)c,+21c[27]	JAK 2 R683
29	PRE-B ALL	Female	4,9	4	47,XX,+21c[30]	WT
30	PRE-B ALL	Female	2,6	18	48,XX,+X,add(18)(q22),+21c[2]/47,XX,+21c[30]	WT
31	C-ALL	Male	13,5	1,2	56,XY,+X,+4,+10,+14,+14,+17,+18,+18,+21c,+mar[13]/47,XY,+21c[9]	WT
32	C-ALL	Male	15,4	4	47,XY,t(1;3)(q32;q26),t(2;12)(q23;q13),t(7;8)(q31;q12),del(13)(q14q32),+21c[17]/40-46,idem[4]	WT
33	PRE-B ALL	Female	4,0	48	NA	WT
34	PRE-B ALL	Female	2,6	9	57,XX,+5,+6,+17,+18,+21c,+5mar[23]/47,XX,+21c[9]	WT
35	C-ALL	Male	4,2	41	46,XY,t(8;9)(q24;p13),del(12)(p13.1),?dic(12;13)(p11.2;p10),+21c[20].ish del(12)(TEL-,AML1-,CEP12+),?dic(12;13)(TEL-,AML1-,CEP12+)	WT
36	PRE-B ALL	Female	2,8	33	47,XX,+21c	WT
37	PRE-B ALL	Male	3,9	78	47,XY,+21c	WT
38	PRE-B ALL	Male	5,6	5	47,XX,+21c	WT
39	C-ALL	Female	7,9	13	47,XX,+21c[21]	WT
40	C-ALL	Male	6,8	199	47,XY,der(9)del(9)(p13p2?2)t(9;22)(q34;q11),+21c,der(22)t(9;22)[15]/47,idem,add(21)(q22)[7]/47,XY,+21c[1]	WT
41	C-ALL	Male	3,7	17	48,XY,+21c,+mar[4]/48,XY,+X,+21c[3]/47,XY+21c[13]	WT
42	C-ALL	Male	17,1	20	47,XY,+21c[20]	WT
43	C-ALL	Female	7,0	33	47,XX,del(12)(p11p13),+21c[18]/47,XX,+21c[2]	WT
44	C-ALL	Female	8,4	4	58~59,XX,+4[3],+6,+10[2],+11,+?14,+18[2],+21,+21c,+1~5mar,inc[cp4]/47,XX,+21c[5]	WT

Table S2 (continued)
Clinical and genetic characteristics of the included DS ALL patients

ID	IPT	Sex	Age	WBC	Karyotype	*JAK*
45	PRO-B ALL	Male	13,3	7	46,XY,-13,+21c[17]/47,XY,+21c[3]	WT
46	C-ALL	Female	13,8	2	49,XX,+X,+5,+21c	WT
47	C-ALL	Male	5,5	5	47,XY,+21c	WT
48	C-ALL	Female	8,1	390	47,XX,-2,-8,+21c,+mar1,+mar2[8]/46,idem,-X/47,XX,+21c[3]	WT
49	PRE-B ALL	Female	5,4	4	47,XX,add(17)(q2?5),+21c[4]/47,XX,+21c[28]	WT
50	PRE-B ALL	Male	4,9	11	47,XY,+21c[32]	WT
51	C-ALL	Female	3,7	9	47,XX,+X,-13,i(17)(q10),der(19)t(13;19)(q1?3;q1?2),+21c[15]/47,idem,del(12)(q1?4q2?1)[7]/52,idem,+3,+10,+14,+21,+21[2]/47,XX,+21c[29]	WT
52	C-ALL	Male	2,3	6	47,XY,+21c	WT
53	PRE-B ALL	Female	4,5	6	47,XX,+21c[32]	WT
54	C-ALL	Female	4,5	5	47 XX +21c [5]	WT

WBC=white blood cell count (*10^9/l) ; WT = wildtype; IPT= immunophenotype; NA = not available.
Age in years. Karyotype nomenclature according to ISCN 1995.

CHAPTER 4

Supplementary Table 1
Hotspot regions, primers and PCR conditions for the investigated genes.

Gene*	Region / Exon	Primer sequences	PCR conditions**
NPM1[1]	exon 12	5′-CTGGTGGTAGAATGAAAAATAGAT-3′ 5′-GGCAGGGACATTCTCATAG-3′	1
CEBPA[2]	whole gene	5′-CGCCATGCCGGGAGAACTCT-3′ 5′-CTTGGCTTCATCCTCCTCGC-3′	2
		5′-CGGCCGCTGGTGATCAAG-3′ 5′-CCAGGGCGGTCCCACAGC-3′	3
MLL[3]	specific PTD's	5′-AGGAGAGAGTTTACCTGCTC-3′ 5′-GGAAGTCAAGCAAGCAGGTC-3′	1
WT1[4]	exon 7	5′-CATGGGGATCTGGAGTGTGA-3′ 5′-TGGGTCCTTAGCAGTGTGAGA-3′	1
	exon 9	5′-TAGGGCCGAGGCTAGACC-3′ 5′-TTCCAATCCCTCTCATCACAAT-3′	1
FLT3[5]	exon 14/15 (ITD)	5′-GCAATTTAGGTATGAAAGCCAGC-3′ 5′-CTTTCAGCATTTTGACGGCAACC-3′	1
	exon 20 (TKD)	5′-TCACCG GTACCTCCTACTG-3′ 5′-AAATGCACCACAGTGAGTG-3′	1
N-RAS[6]	exon 2	5′-GGGGGTTGCTAGAAAACTA-3′ 5′-ATCCGACAAGTGAGAGACA-3′	1
	exon 3	5′-CCCAGGATTCTTACAGAAAA-3′ 5′-TCCCCATAAAGATTCAGAAC-3′	1
K-RAS[6]	exon 2	5′-CGTCGATGGAGGAGTTT-3′ 5′-AACCCAAGGTACATTTCAGA-3′	1
PTPN11[6]	exon 3	5′-TTGGGTTTCTTTCAACACTT-3′ 5′-GCCTTTGGAGTCAGAGAGT-3′	1
	exon 13	5′-TGGCTCTGCAGTTTCTCT-3′ 5′-CATTCCGAAATCAAACAGTT-3′	1
KIT[6]	exon 8	5′-CCGCCTCCTTGTACCTT-3′ 5′-TTCAGCAAACAAAATTAATGTCTA-3′	4
	exon 17	5′-TCCTCCAACCTAATAGTGTATTC-3′ 5′-CATTCCGAAATCAAACAGTT-3′	4
IDH1	exon 4	5′-TTGGGAATTGATTAAAGAGAAA-3′ 5′-CACCACCTTCTTCAAAGTTATG-3′	1
IDH2	exon 4	5′-AACCGTGACCAGACTGATG-3′ 5′-GTTCTTGGTGCTCATGTACAG-3′	1
DNMT3A[7]	exon 12-23	5′-GCCACCAGAAGAAGAGAAGA-3′ 5′-GCACCTGCAGCAGTTGT-3′	1
		5′-CCGGAACATTGAGGACA-3′ 5′-CTGGGACAGGTGGGTAAA-3′	1
		5′-CGGAGGTGTGTGAGGACT -3′ 5′-GTTCATACCGGGAAGGTTAC-3′	1
		5′-CTCCAGATGTTCTTCGCTAA-3′ 5′-CCACTCCTGGATATGCTTCT-3′	1

* the superscript numbers refer to the below mentioned papers in which the methods were fully described.

** numbers refer to the following PCR programs:

1 10' 95°C, 40 cycles of 1' 95°C and 1' 60°C, 10' 72°C
2 10' 95°C, 35 cycles of 1' 95°C, 1' 60°C and 1' 72°C, 10' 72°C
3 10' 95°C, touchdown 20 cycles of 1' 95°C, 1' 70-60°C and 1' 72°C plus 20 cycles of 1' 95°C, 1' 60°C and 1' 72°C, 10' 72°C
4 ' 95°C, touchdown 20 cycles of 1' 95°C, 1' 66-56°C and 1' 72°C plus 14 cycles of 1' 95°C, 1' 56°C and 1' 72°C, 10' 72°C

[1] Hollink IH, Zwaan CM, Zimmermann M, et al. Favorable prognostic impact of NPM1 gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML. Leukemia 2009;23:262-70.
[2] Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, et al. Characterization of CEBPA mutations and promoter hypermethylation in pediatric acute myeloid leukemia. Haematologica.2011;96:384-92.
[3] Balgobind BV, Hollink IH, Reinhardt D, et al. Low frequency of MLL-partial tandem duplications in paediatric acute myeloid leukaemia using MLPA as a novel DNA screenings technique. Eur J Cancer 2010;46:1892-9.
[4] Hollink IH, van den Heuvel-Eibrink MM, Zimmermann M, et al. Clinical relevance of Wilms tumor 1 gene mutations in childhood acute myeloid leukemia. Blood 2009;113:5951-60.
[5] Stam RW, den Boer ML, Schneider P, et al. Targeting FLT3 in primary MLL-gene-rearranged infant acute lymphoblastic leukemia. Blood. 2005;106:2484-90.
[6] Balgobind BV, Van Vlierberghe P, van den Ouweland AM, et al. Leukemia-associated NF1 inactivation in patients with pediatric T-ALL and AML lacking evidence for neurofibromatosis. Blood 2008;111:4322-8.
[7] Hollink IH, Feng Q, Danen-van Oorschot AA, Arentsen-Peters ST, Verboon LJ, Zhang P, et al. Low frequency of DNMT3A mutations in pediatric AML, and the identification of the OCI-AML3 cell line as an in vitro model. Leukemia. 2011 Aug 12.

CHAPTER 5

See Color Figures page 163

CHAPTER 6

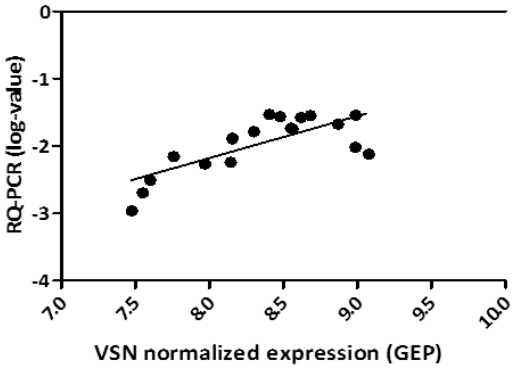

Figure S1
Correlation between gene expression profiling and RQ-PCR for *MLLT3* expression.
Log transformed data of gene expression profiling and RQ-PCR showed high a high correlation with r_s=0.7 for *MLLT3* expression; p-value 0.0012.

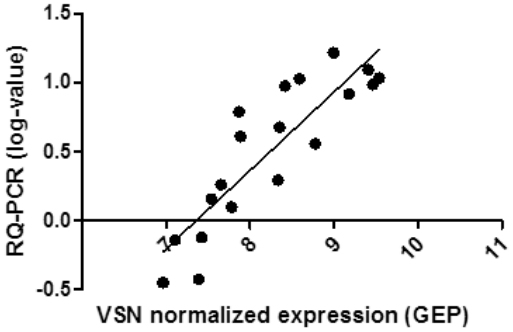

Figure S2
Correlation between gene expression profiling and RQ-PCR for *ITGA4* expression.
Log transformed data of gene expression profiling and RQ-PCR showed high a high correlation with r_s=0.9 for ITGA4 expression; p-value < 0.001.

Table S1:
Primers sequences PCR

Primer	Sequence (5'- 3')
MLL forward	CGT CGA GGA AAA GAG TGA
MLLT3 reverse	ATG TTT CCA GGT AAC TCT GTA GT

Table S2:
Primers sequences RQ-PCR

Primer	Sequence (5'- 3')
MLLT3 forward	AAG CAA AGC AAA TCA GAT AAG
MLLT3 reverse	CAG CGA GCA AAG ATC AA
ITGA4 forward	GCC AAC GCT TCA GTG A
ITGA4 reverse	GGC ACT CCA TAG CAA CC
IGF1R forward	CAA GGG TGT GGT GAA AGA
IGF1R reverse	CGG CCA TCT GAA TCA TC
GAPDH forward	GTC GGA GTC AAC GGA TT
GAPDH reverse	AAG CTT CCC GTT CTC AG

Table S3:
Primers sequences mutational screening by sequencing

Primer	Sequence (5'-3')
*MLLT3*_1 forward	ACA CCC CAG CAA ACC TC
*MLLT3*_1 reverse	CTG CCT TCA GCA ACT TTC T
*MLLT3*_2 forward	GGC CAT CCA CCA GTG A
*MLLT3*_2 reverse	TTT GGG TTT CTT AGA GGA TTC T
*MLLT3*_3 forward	CCA GGG ATC ACA ACA AAT C
*MLLT3*_3 reverse	GAC TGG GTT GTT CAG AAT CA
*MLLT3*_4 forward	ATC CCA ATG ATT CAG ATG TG
*MLLT3*_4 reverse	GGT TGC ATC ATT TTG AGT GT

COLOR FIGURES

Chapter 1

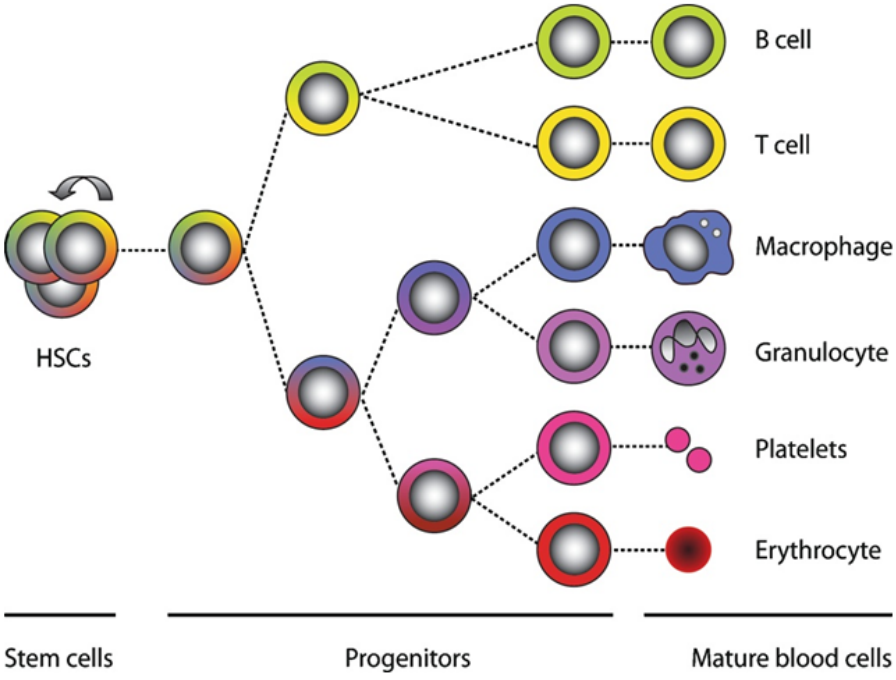

Figure 1. Schematic overview of hematopoiesis.
HSC= hematopoietic stem cell

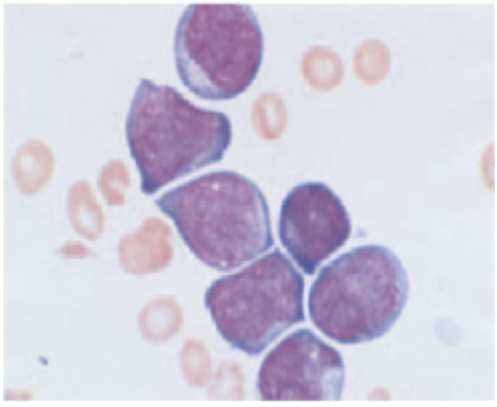

Figure 2.
Blasts in peripheral blood
(May-Grünwald-Giemsa staining)

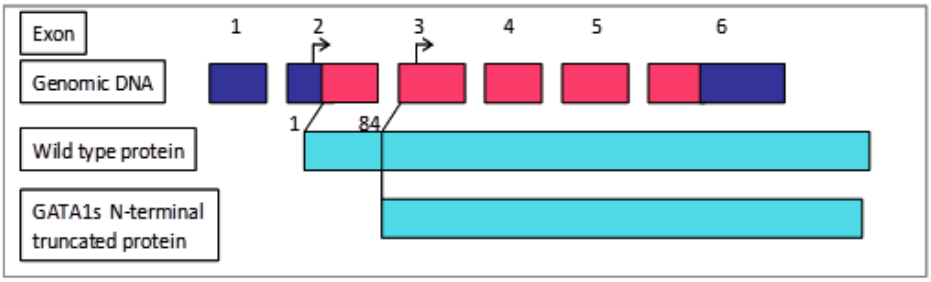

Figure 3.
Schematic overview of mutations in the *GATA1*-gene.

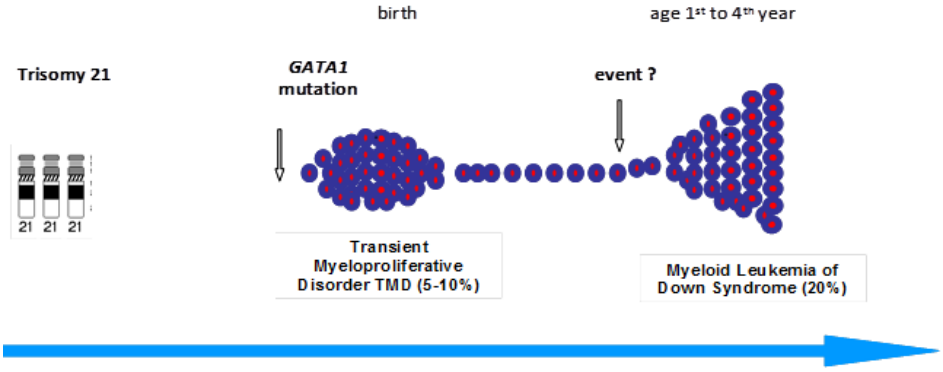

Figure 4.
Schematic overview of ML-DS leukemogenesis.

Chapter 2

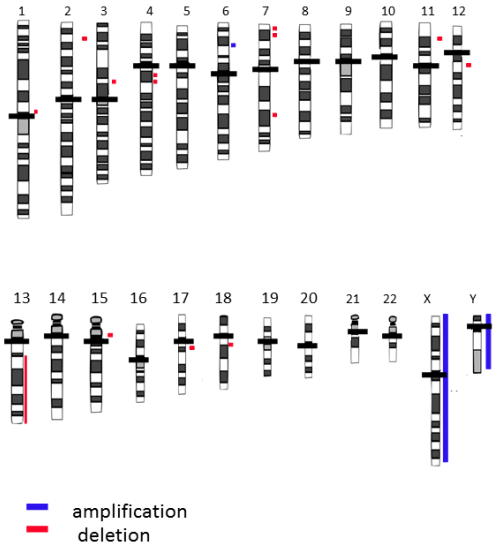

Figure 1A
Schematic overview of all CNAs taken together in TMD patients (n = 7).

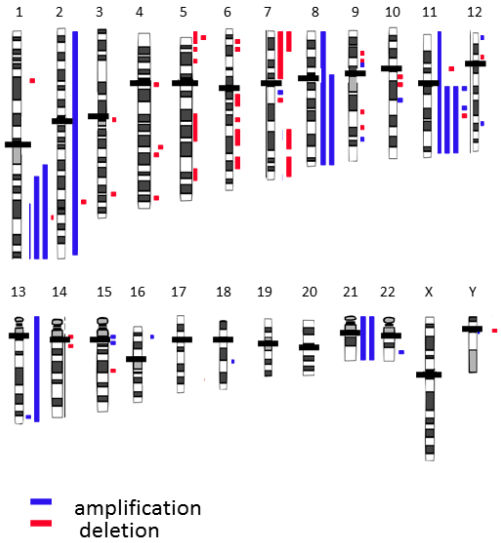

Figure 1B
Schematic overview of all CNAs taken together in ML-DS patients (n = 14).

Chapter 3

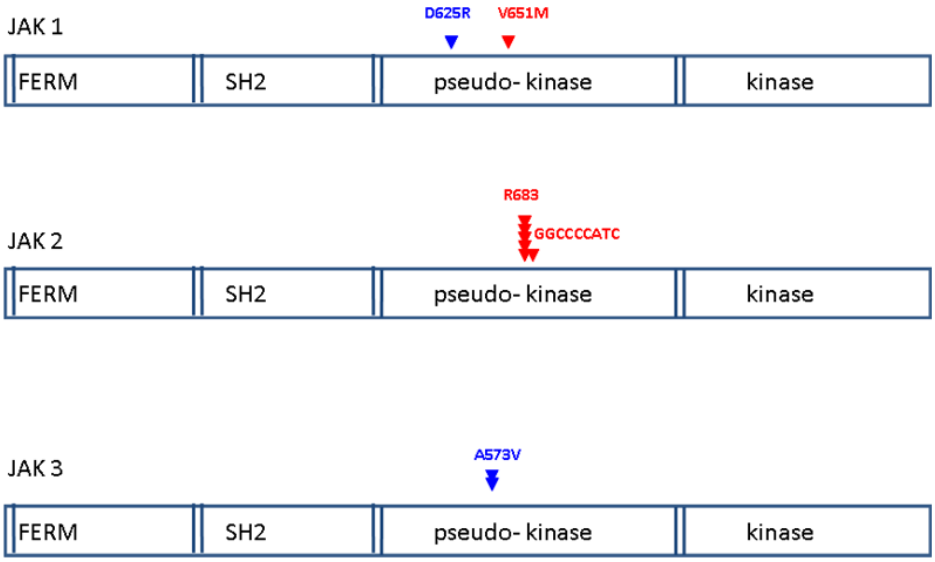

Figure 1
Localization of mutations in JAK 1-3. Schematic overview of the structure of *JAK 1-3* with the kinase, pseudokinase and FERM (4.1-ezrin-radixin-moesin) domain in which the location of the mutations we identified is indicated. Mutations in DS ALL are depicted in red, mutations in ML-DS and TMD in blue.

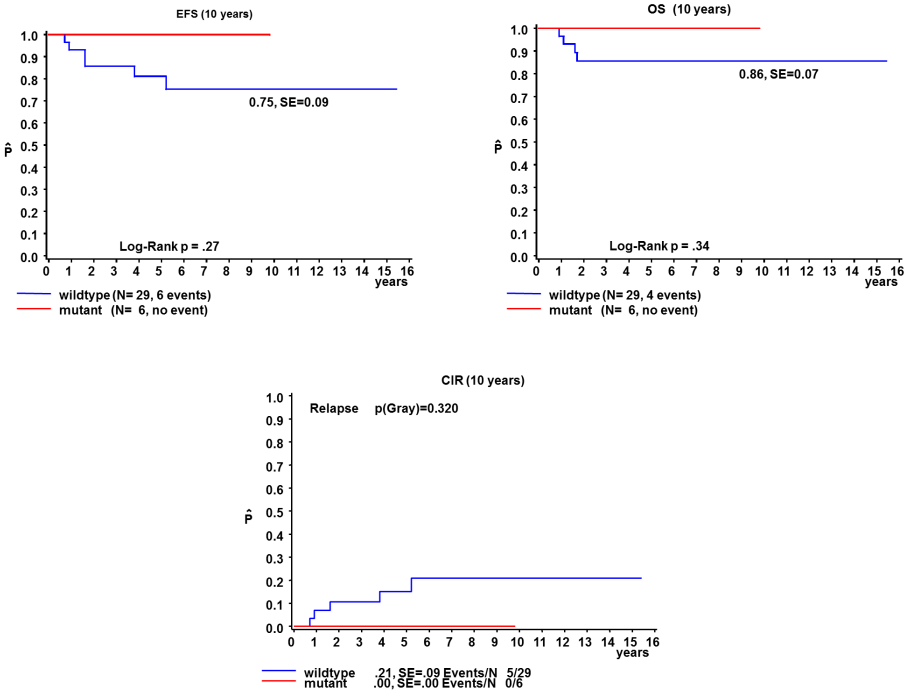

Figure 2

The 10-year survival parameters of *JAK2* R683 mutations in DS ALL patients.

(a) EFS (100% vs. 75±9%; *P*=0.27)

(b) OS (100% vs. 86±7%; *P*=0.30)

(c) CIR (0% vs. 21±9%; *P*=0.32)

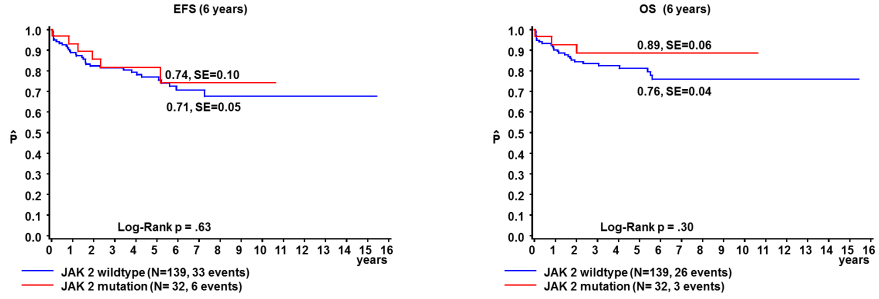

Figure 3

The 6-year survival parameters of *JAK2* mutant and wild type patients; meta-analysis of three datasets. DS ALL patients were diagnosed between 1992 and 2008.

(a) EFS 74±10% vs. 71±5%; *P*=0.63

(b) OS 89±6% vs. 76±4%; *P*=0.30

Chapter 5

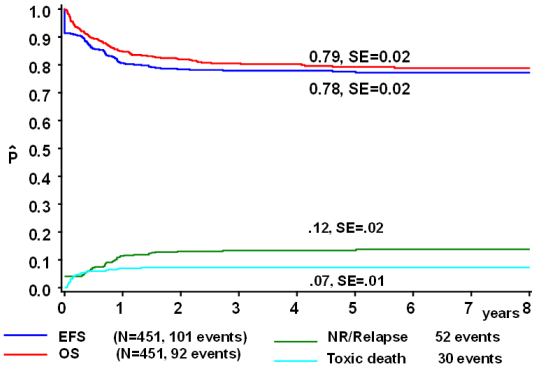

Figure 2: Survival curves of all 451 ML-DS patients included in this study.
The 7-yr overall survival (OS) was 79% (±2%); the 7-yr event-free survival (EFS) 78% (±2%); the 7-yr cumulative incidence of relapse (CIR) was 12% (±2%); and the cumulative incidence (CI) of toxic death at 1.5 years from diagnosis was 7% (±1%). NR= non remitters.

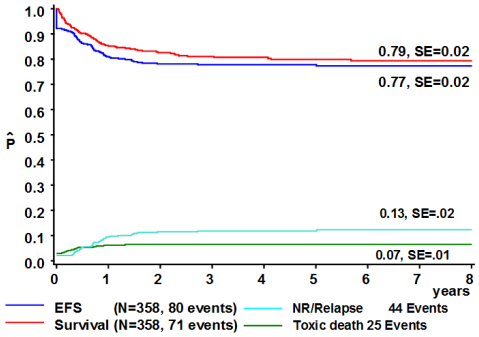

Figure 3: Survival curves of the 358 ML-DS patients with evaluable karyotypes
The 7-yr overall survival (OS) was 79% (±2%); the 7-yr event-free survival (EFS) 77% (±2%); the 7-yr cumulative incidence of relapse (CIR) was 13% (±2%); and the cumulative incidence (CI) of toxic death at 1.5 years from diagnosis was 7% (±1%). NR= non remitters.

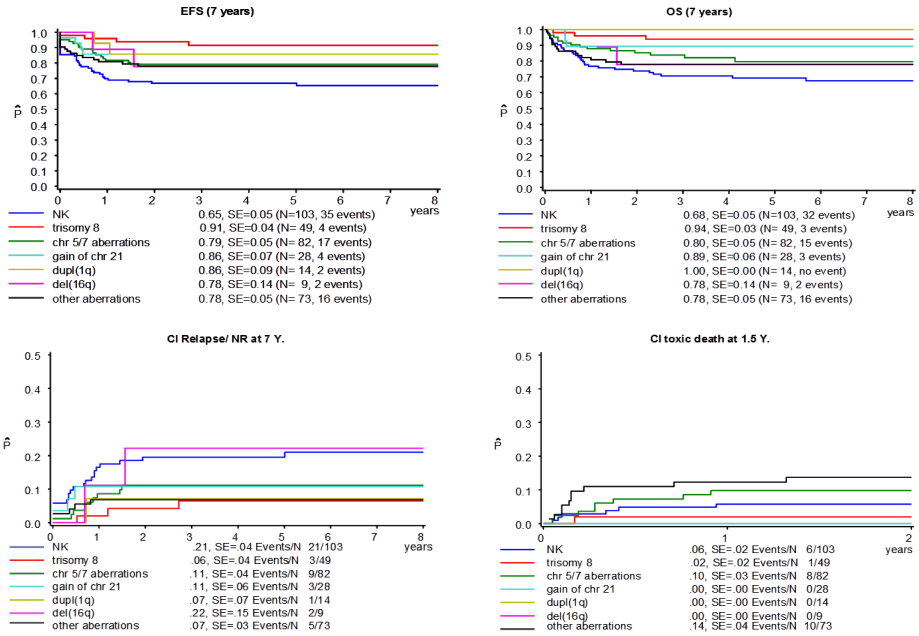

Figure 4

Survival curves for ML-DS patients (n=358) based on their cytogenetic status

A. Event-free survival curves

B. Overall survival curves

C. Cumulative incidence of relapse

D. Cumulative incidence of toxic death

Assignment to groups was based on cytogenetic status, as identified after central reviewing.

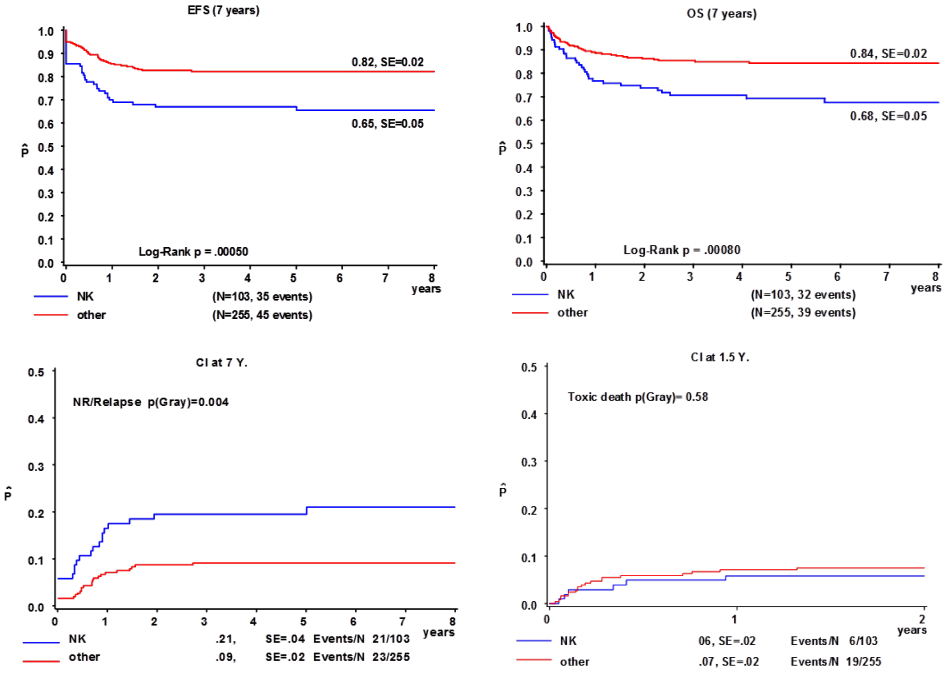

Figure 5

Survival curves for NK ML-DS patients (n=103) versus all other patients (n=255).

A. Event-free survival curves

B. Overall survival curves

C. Cumulative incidence of relapse

D. Cumulative incidence of toxic death

Assignment to groups was based on cytogenetic status, as identified after central reviewing.

Supplementary data chapter 5

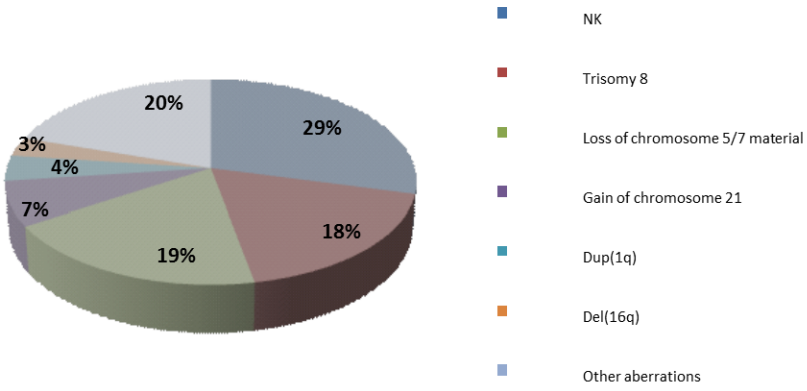

Figure S1
Distribution of cytogenetic subgroups within ML-DS
NK= normal karyotype; del= deletion; dupl= duplication

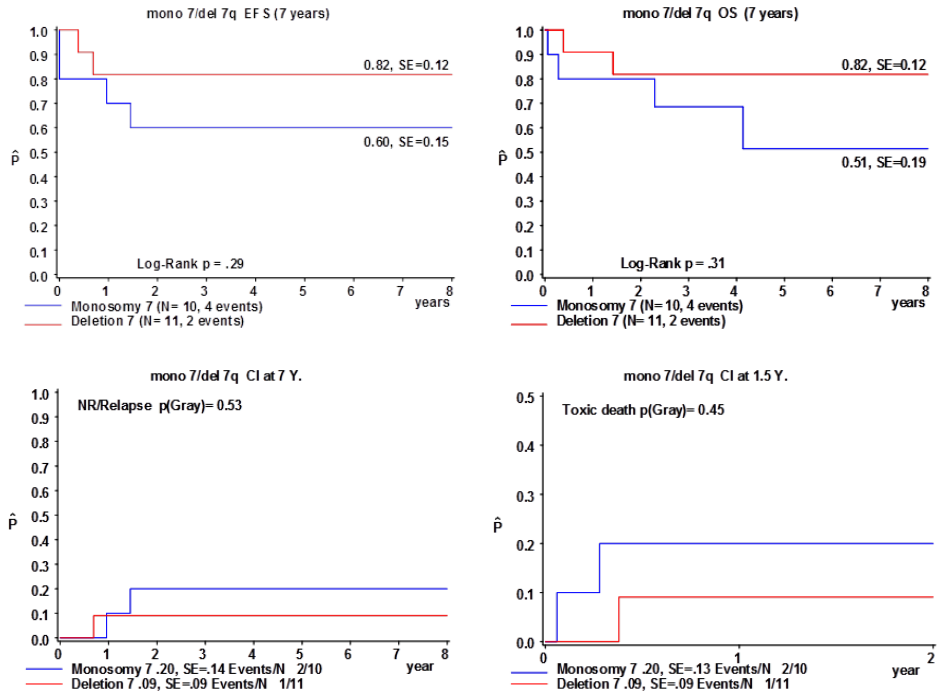

Figure S2: Survival curves of the subgroups of monosomy 7 patients (n=10) and patients with a deletion of 7q (n=11).

A. Event-free survival curves
B. Overall survival curves
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

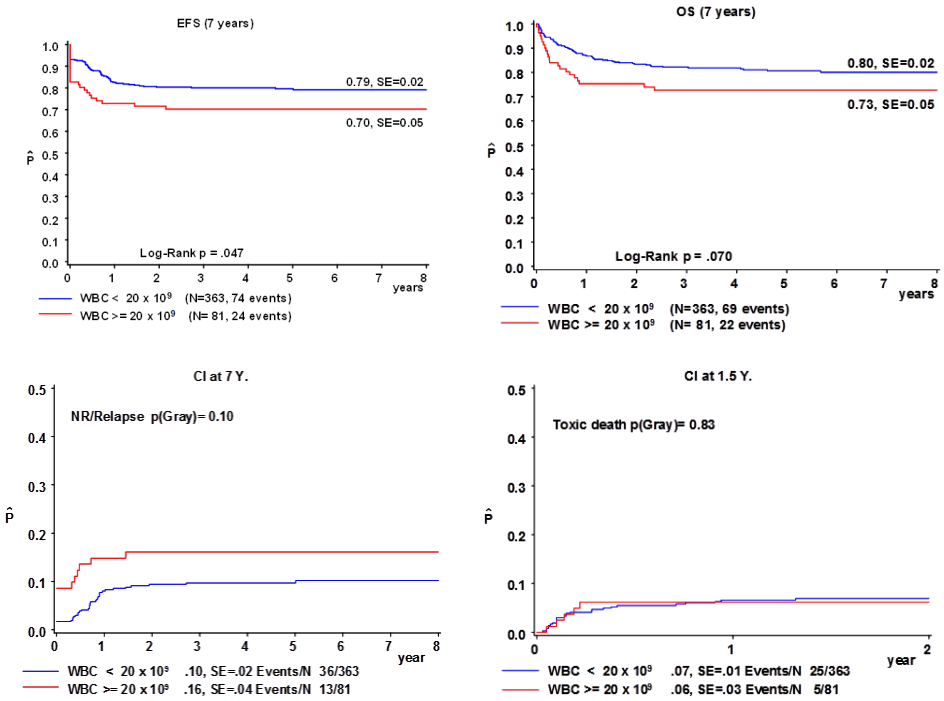

Figure S3: Survival curves of ML-DS patients (n=444) according to white blood cell count (WBC).

A. Event-free survival
B. Overall survival
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

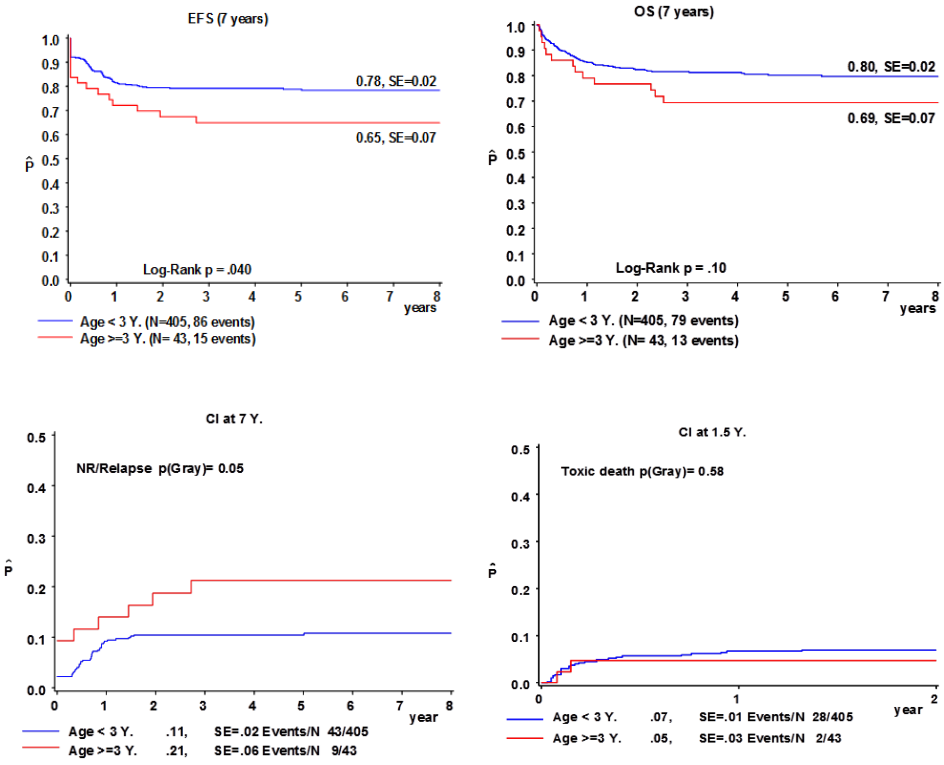

Figure S4: Survival curves for ML-DS (n= 448) patients according to age.

A. Event-free survival
B. Overall survival
C. Cumulative incidence of relapse
D. Cumulative incidence of relapse

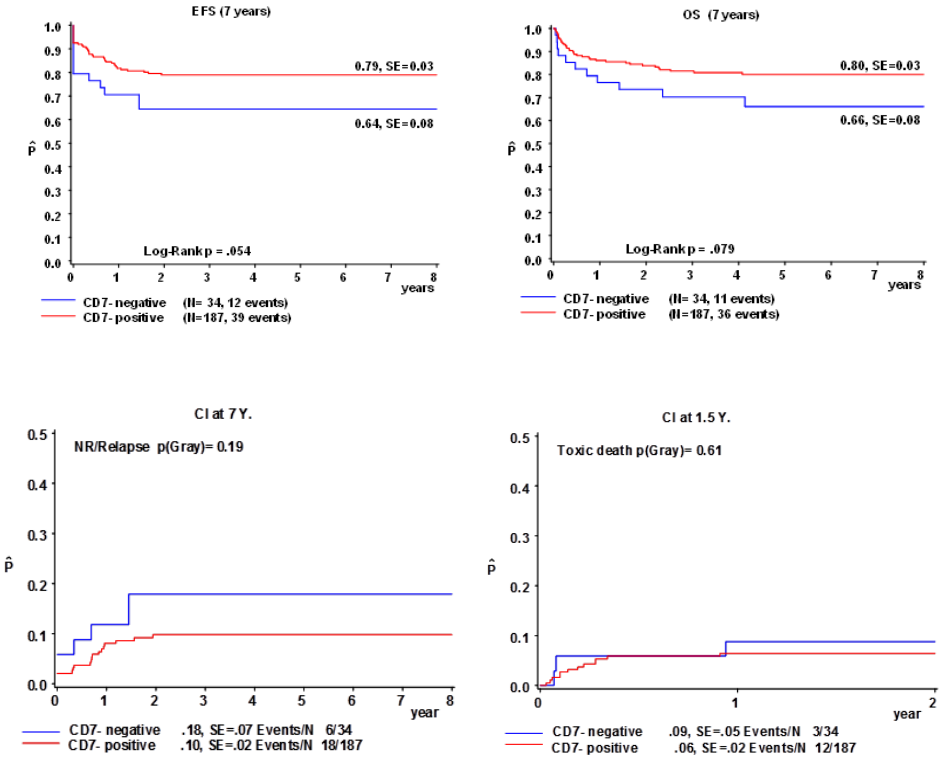

Figure S5: Survival curves for ML-DS patients (n=221), positive and negative for CD7

A. Event-free survival curves
B. Overall survival curves
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

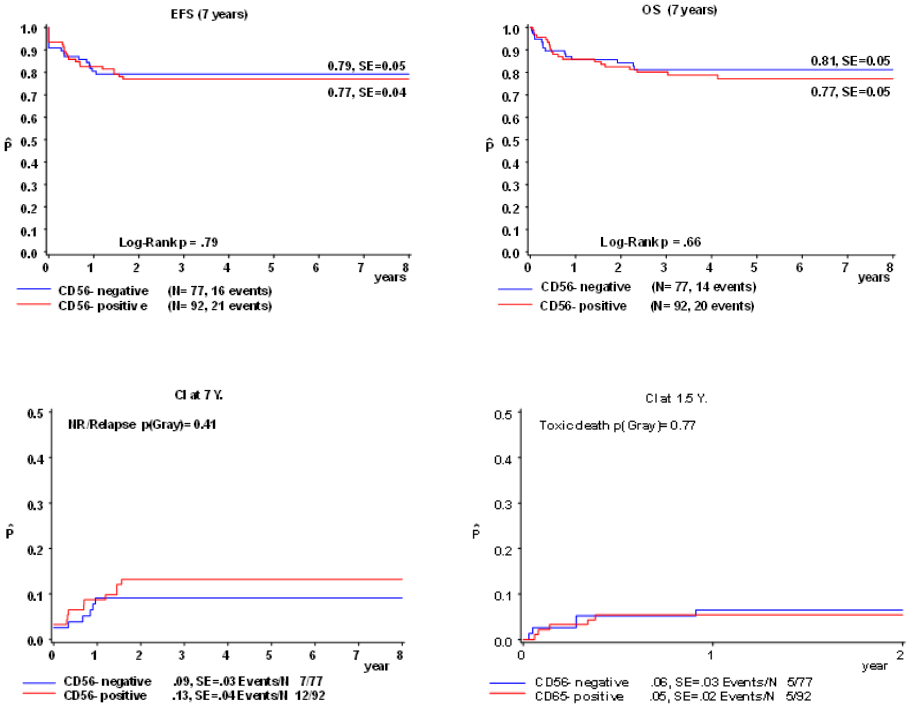

Figure S6: Survival curves for ML-DS patients (n=169), positive and negative for CD56

A. Event-free survival curves
B. Overall survival curves
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

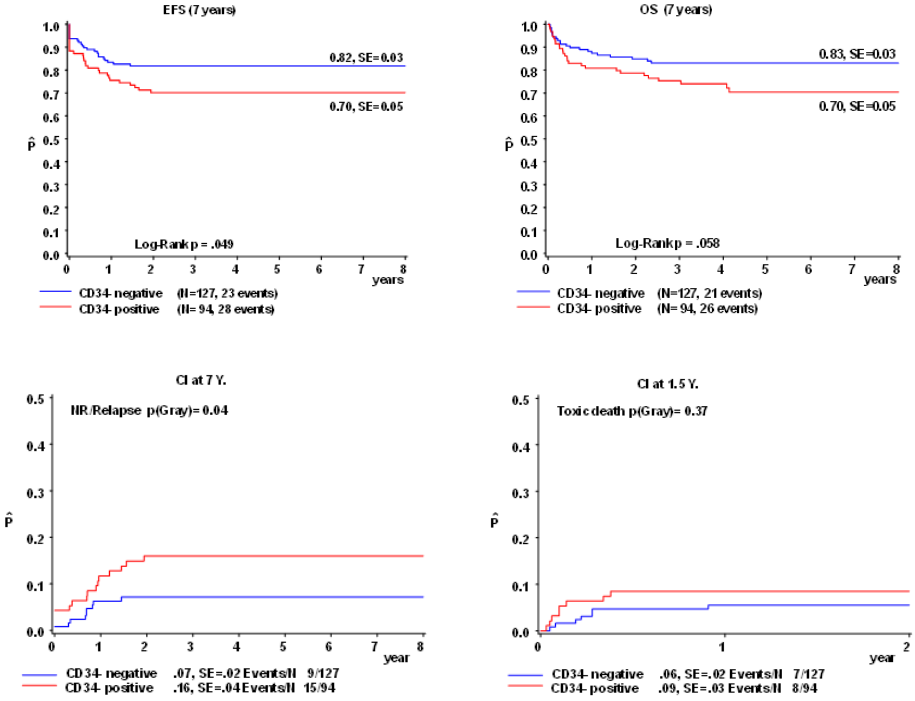

Figure S7: Survival curves for ML-DS patients (n=221), positive and negative for CD34

A. Event-free survival curves
B. Overall survival curves
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

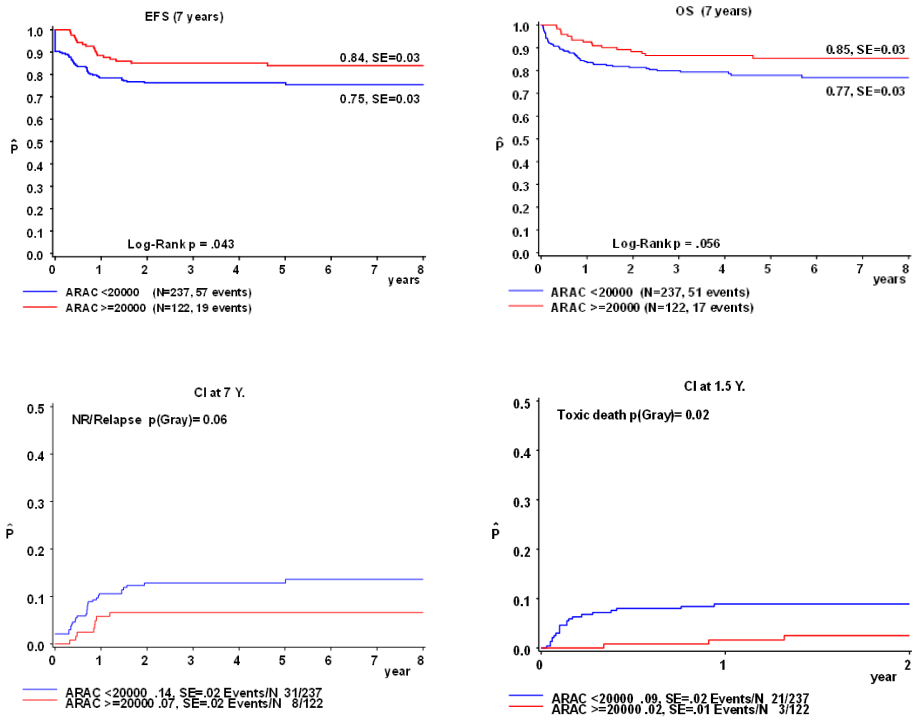

Figure S8: Survival curves for ML-DS patients (n=359) treated with different dosages of cytarabine (Ara-C)

A. Event-free survival curves
B. Overall survival curves
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

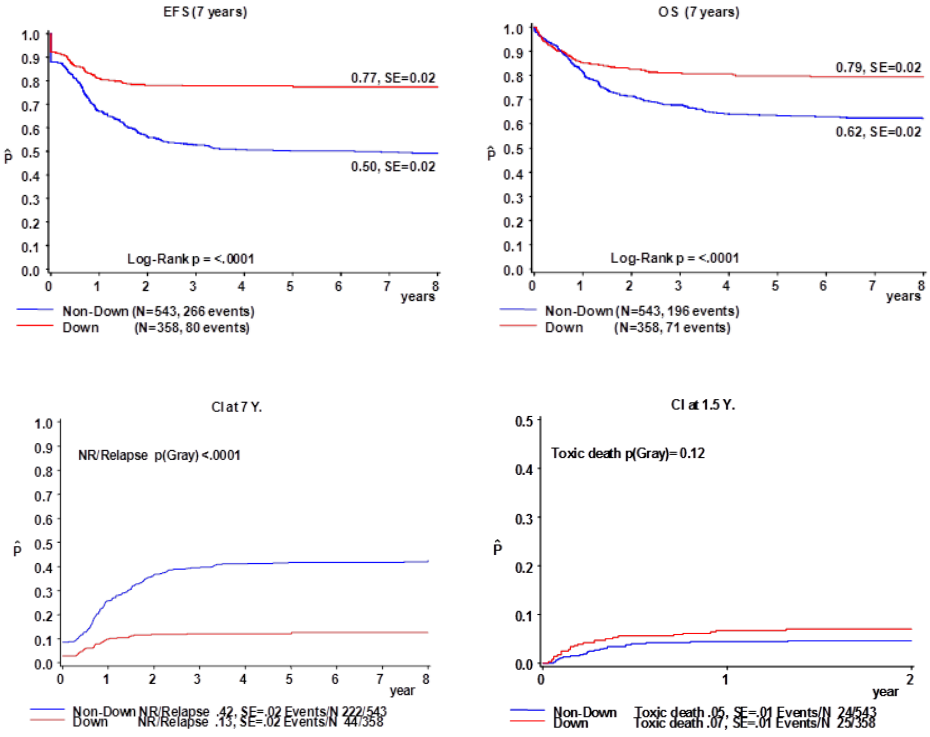

Figure S9: Survival curves for non- DS AML patients (n=543) compared to ML-DS patients (n=358)

A. Event-free survival curves
B. Overall survival curves
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

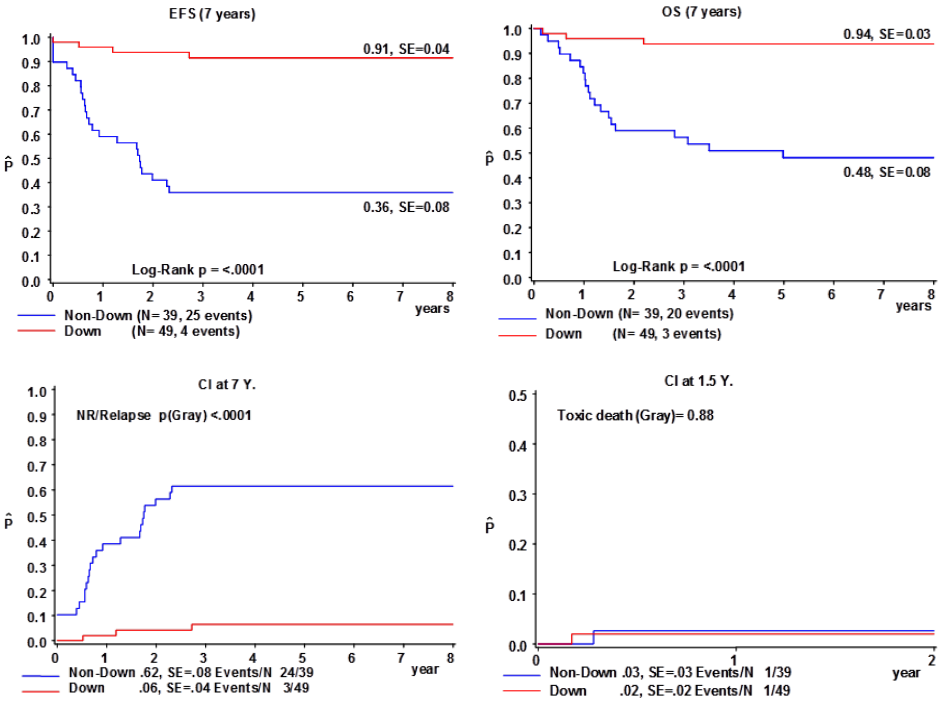

Figure S10: Survival curves for trisomy 8 patients; non-DS AML (n=39) vs. ML-DS (n=49)

A. Event-free survival curves
B. Overall survival curves
C. Cumulative incidence of relapse
D. Cumulative incidence of toxic death

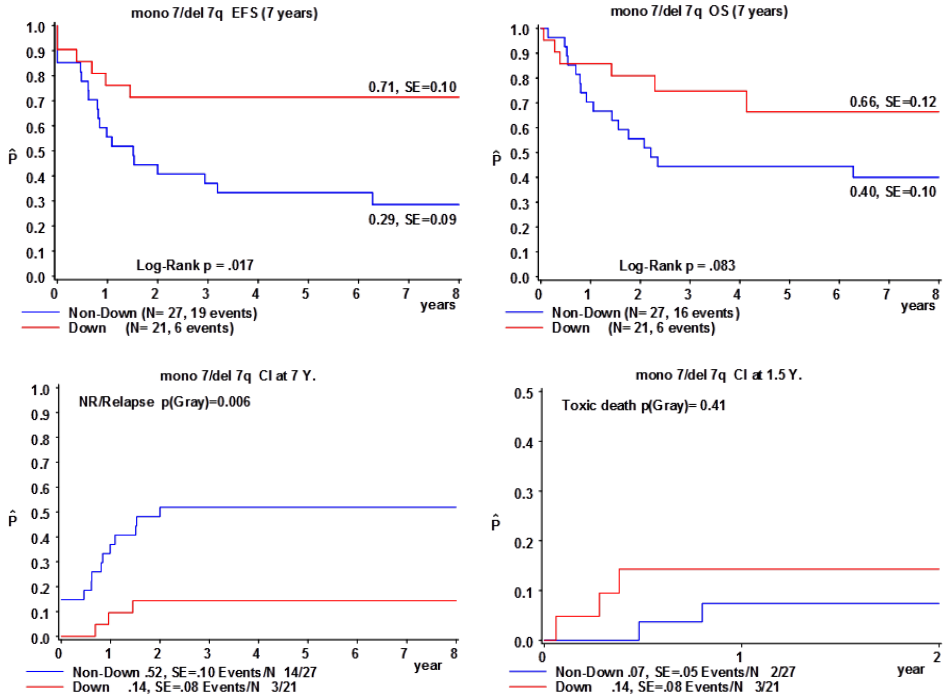

Figure S11: Survival curves for patients with monosomy 7/ del 7q; non-DS AML (n=27) vs. ML-DS (n=21)

A. **Event-free** survival curves

B. **Overall** survival curves

C. **Cumulative** incidence of relapse

D. **Cumulative** incidence of toxic death

Chapter 6

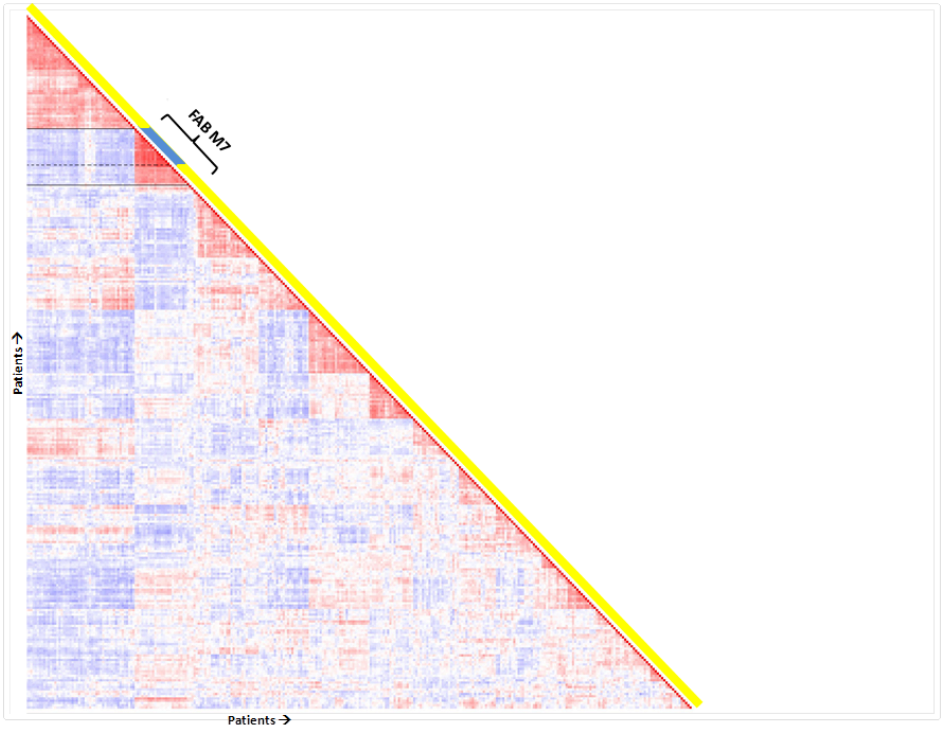

Figure 1
Unsupervised clustering of gene expression data revealed clustering of the ML-DS cases in the non-DS M7 pediatric leukemias.
Pair wise correlations between gene expression profiles of 297 *de novo* pediatric AML and 14 ML-DS samples, are displayed in a correlation plot. Colors of boxes represent the Pearson's correlation coefficient with a color gradient ranging from deep blue for a negative correlation, to vivid red for a positive correlation. Distinct clusters of samples, which can be recognized by the red blocks showing high correlation along the diagonal, are observed. The column to the right of the plot indicates the ML-DS patients (in blue), clustering all together within the non-DS AML FAB M7 leukemias.

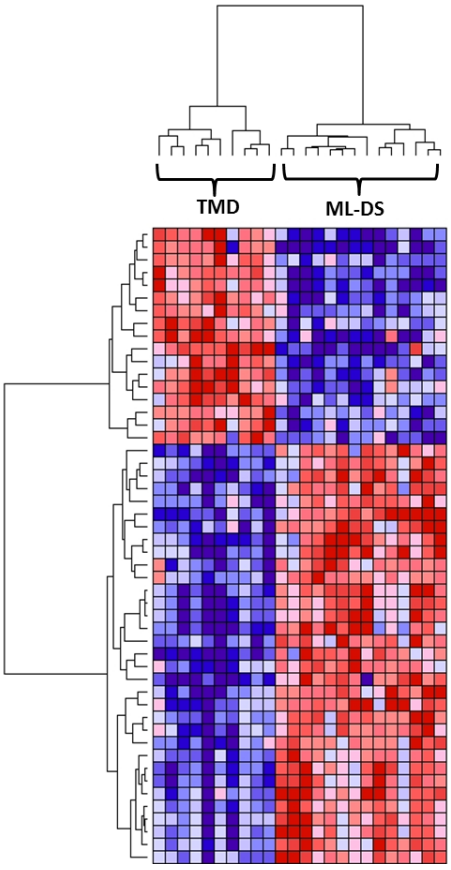

Figure 2
Hierarchical clustering based on the top 50 most discriminative genes for TMD and ML-DS patients. Hierarchical clustering of 24 TMD and ML-DS cases (columns) based on their top 50 most discriminative genes (rows) (Table 2).

ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMKL	Acute megakaryoblastic leukemia
A-CGH	Array-based comparative genome hybridization
BFM-SG	Berlin-Frankfürt-Münster Study Group
BM	Bone marrow
CD	Cluster of differentiation
CEBPA	CAAT/enhancer binding protein alpha (gene)
CIR	Cumulative incidence of relapse
CNA	Copy number alteration
CN-AML	Cytogenetically normal AML
CNS	Central nervous system
CR	Complete remission
DCOG	Dutch Childhood Oncology Group
DNA	Deoxyribonucleic acid
DS	Down syndrome
EFS	Event-free survival
ERG	v-ETS erythroblastosis virus E26 oncogene homolog (gene)
FAB	French-American-British
FISH	Fluorescent *in situ* hybridization
FLT3/ITD	FMS-like tyrosine kinase 3 (gene) internal tandem duplication
FLT3/TKD	FLT3 tyrosine kinase domain mutation
GEP	Gene expression profiling
HR	Hazard ratio
HSC	Hematopoietic stem cell
IDH1/2	Isocitrate dehydrogenase 1/2(gene)
ITGA4	Integrin alpha 4 gene (gene)
JAK1/2/3	Janus kinase (gene)
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene (gene)
K-RAS	v-kit ras2 Kirsten rat sarcoma viral oncogene homolog (gene)
LIMMA	Linear models for micro-array data
ML-DS	Myeloid leukemia of Down syndrome
MLL-PTD	Mixed lineage leukemia (gene) partial tandem duplication
MLLT3	Myeloid/lymphoid or mixed-lineage leukemia, translocated to 3 (gene)
mRNA	Messenger RNA
NK	Normal karyotype
NPM1	Nucleophosmin (gene)

N-RAS	Neuroblastoma RAS viral (v-ras) oncogene homolog (gene)
NUP98	Nucleoporin 98kD (gene)
NSD1	Nuclear receptor binding SET domain protein 1 (gene)
OS	Overall survival
PCR	Polymerase chain reaction
PTPN11	Protein tyrosine phosphatase, non-receptor type 11 (gene)
RNA	Ribonucleic acid
RFS	Relapse-free survival
RT-PCR	Reverse transcription PCR
RT-qPCR	Real-time quantative PCR
siRNA	Small interfering RNA
SCT	Stem cell transplantation
SNP	Single nucleotide polymorphism
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TMD	Transient myeloproliferative disorder
VSN	Variance and stabilization normalization
WHO	World Health Organization
WBC	White blood cell count
WT1	Wilms tumor 1 (gene)

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