MOLECULAR DETERMINANTS OF JUVENILE MYELOMONOCYTIC LEUKEMIA AND CHILDHOOD MYELODYSPLASTIC SYNDROME

Moleculaire afwijkingen van juveniele myelomonocytaire leukemie en myelodysplastisch syndroom op de kinderleeftijd

door

Andrica de Vries

Molecular determinants of Juvenile Myelomonocytic Leukemia and childhood Melodysplastic Syndrome

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wat wij weten is een druppel,
wat wij niet weten een oceaan

what we know is a drop,
what we don't know an ocean

Sir Isaac Newton
1642-1727

Voor mijn ouders
Voor Kees, Elke, Hanne* en Ward

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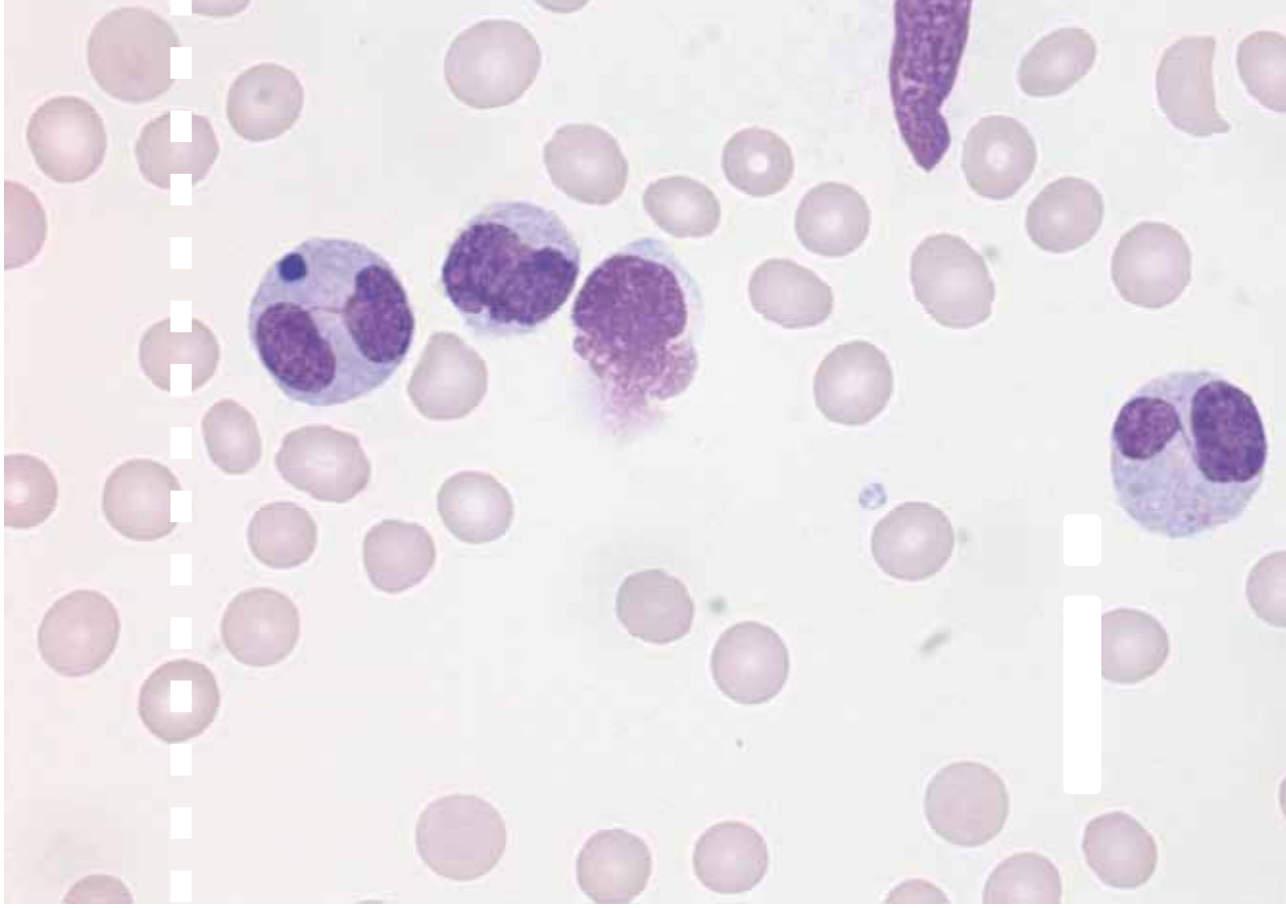

GENERAL INTRODUCTION

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Illustration: JMML monocytosis and abnormal monocytes and phagocytosis (PB)

THE EPIDEMIOLOGY OF PEDIATRIC CANCER

In the general population the probability of developing cancer before the age of 18 years is around 1 in 400. In the Netherlands, approximately 600 new children each year are diagnosed with cancer [1] [Figure 1]. The most common types of childhood cancer are leukemias [1] and the distribution of cancer types varies with age [2-3]. Figure 2 gives an overview of the incidence of the different subclasses of leukemia as registered by the DCOG [2005-2010]. Acute lymphoblastic leukemia [ALL] accounts for about 80% of the leukemias, whereas acute myeloid leukemia [AML] accounts for about 15% of all leukemias. Myelodysplastic syndrome [MDS] and Juvenile Myelomonocytic Leukemia [JMML], which are the subject of this thesis, represent very rare myeloid malignancies in childhood [4-5].

Over the last decades, following better treatment stratification, better chemotherapy combinations and improved supportive care regimens, the 5-year survival of cancer in children and adolescents improved significantly [1] [Figure 3]. The survival of MDS and JMML however, has reached a plateau at approximately 50% following stem cell transplantation, which is the only curative treatment option for these diseases.

HEMATOPOIESIS AND LEUKEMIA

Hematopoiesis [from Ancient Greek: αἷμα, "blood"; ποιεῖν "to make"] is the formation of blood cellular components. All cellular blood components are derived from haematopoietic stem cells [HSCs] and these cells have the unique ability to give rise to

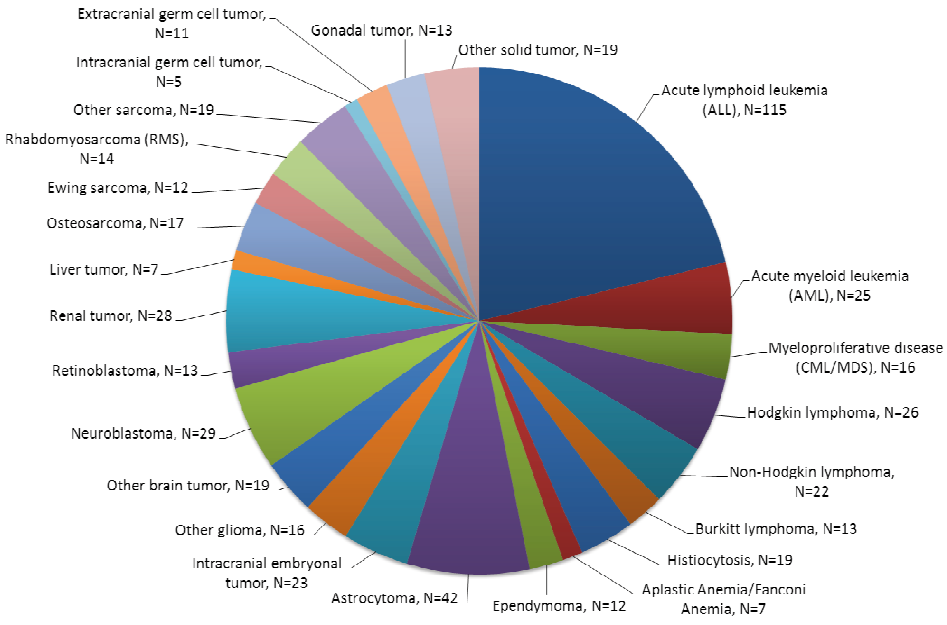


Figure 1: Incidence of childhood cancer per year in The Netherlands [DCOG registration 2005-2010].

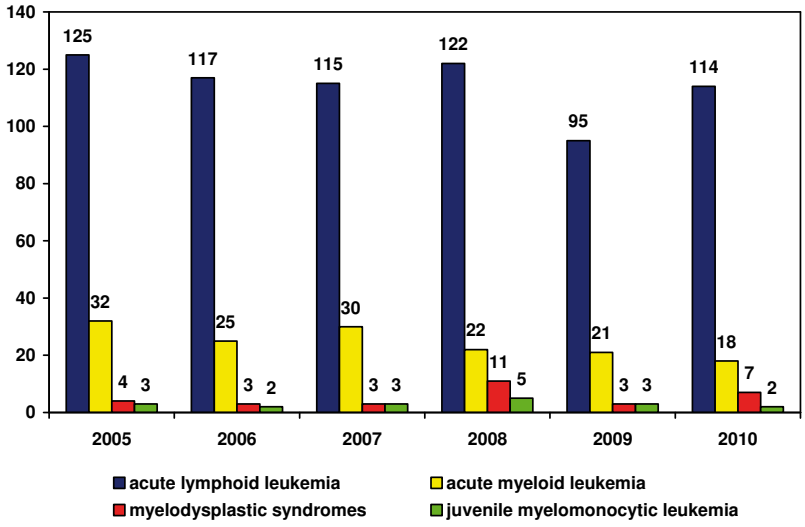


Figure 2: Incidence of hematological malignancies per year in The Netherlands (DCOG registration 2005-2010).

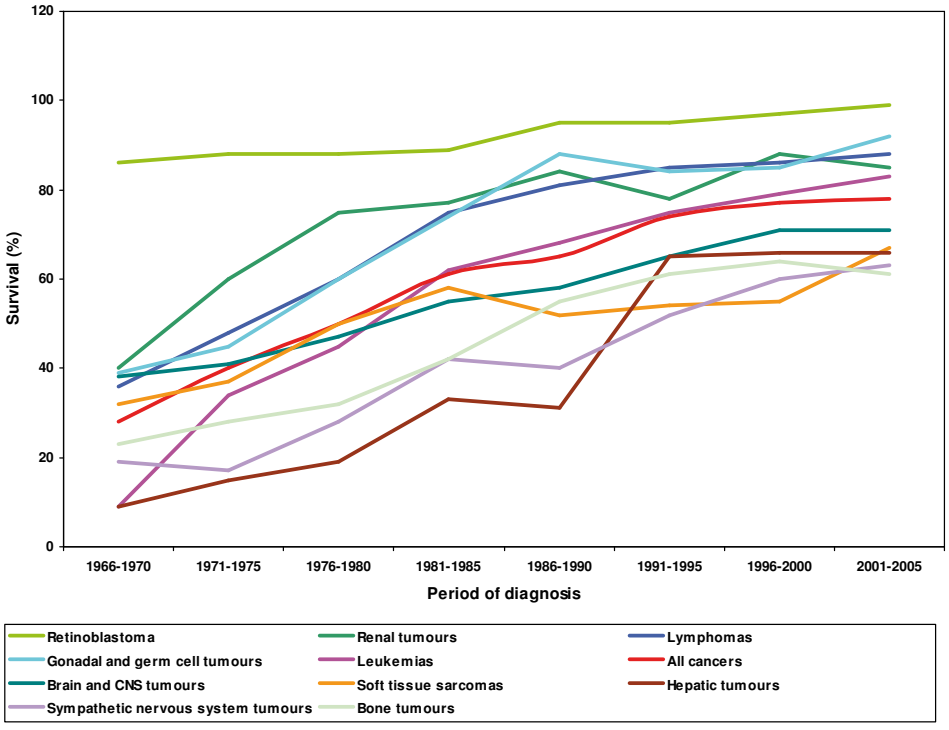


Figure 3: Five year survival by cancer type in children aged 0-14 years (adapted from C. Stiller, Oxford University Press, 2007).

all different mature blood cell types. Blood cells are divided into three lineages: erytroid cells (erythropoiesis), white blood cells (granulocytes, lymphocytes and macrophages) (myelopoiesis) and platelets (megakaryopoiesis) (Figure 4).

Leukemia (from the Greek λεύκος "white", and αίμα "blood") is cancer of the blood, and is characterized by uncontrolled proliferation of a subset of blood cells in the bone marrow, in case of ALL proliferation of the lymphoid lineage and in case of AML proliferation of the myeloid lineage. Two other types of hematological malignancies in childhood are MDS and JMML. According to the WHO classification these diseases are classified as myelodysplastic syndromes and myeloproliferative disorders in childhood (Table 1) (6-7).

The exact pathogenesis of MDS and JMML has not been unraveled. The origin of MDS is a dysregulated stem cell, and therefore all haematopoietic cell lineages are affected (8-10) (Figure 6). In JMML, the monoblastic cell line is dysregulated at an early stage leading to a hyperproliferative disease (Figure 5).

CLINICAL CHARACTERISTICS AND DIAGNOSTICS OF JMML

Clinical characteristics:

JMML is a rare clonal myeloproliferative disorder that according to the WHO classification falls under the myelodysplastic / myeloproliferative diseases (7). With this new classification the old terms juvenile chronic myeloid leukemia (jCMML) and chronic myelomonocytic leukemia (CMMoI) disappeared (11).

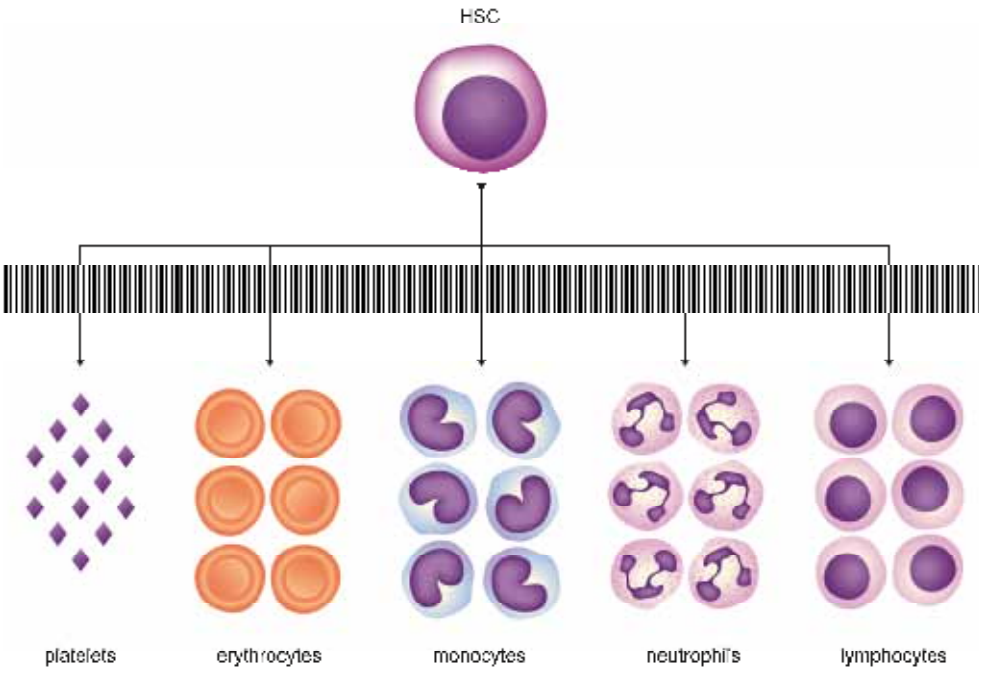


Figure 4: Normal hematopoiesis.

Table 1: Classification Myelodysplastic Syndrome and JMML.

A. WHO classification MDS
Refractory cytopenia with unilineage dysplasia
Refractory anemia with ringsideroblasts
Refractory cytopenia with multilinear dysplasia
Refactory anemia with excess of blasts-1
Refactory anemia with excess of blasts-2
MDS, unclassified
MDS with isolated del(5q)

B. Modified WHO classification: MDS and myeloproliferative disorders in childhood
Myelodysplastic/myeloproliferative disorders
Juvenile myelomonocytic leukemia (JMML)
Chronic myelomonocytic leukemia (CMML)
BCR-ABL negative chronic myeloid leukemia (Ph^- CML)
Myelodysplastic syndrome (MDS)
Refractory cytopenia of childhood (RCC): PB <2% blasts, and BM < 5% blasts
Refractory anemia with excess of blasts (RAEB): PB 2-19% blasts, or BM 5-19% blasts
RAEB in transformation (RAEB-t): PB or BM: 20-29% blasts

1A: WHO classification of MDS for adults (7)
1B: Modified WHO classification for MDS and JMML in childhood (6-7)
PB = peripheral blood
BM = bone marrow

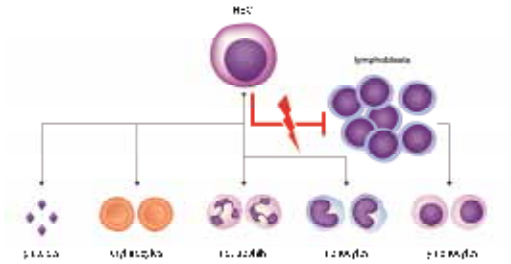
A combined population study in Denmark and Canada showed an incidence of JMML of 1.2 per million children aged 0-14 years. In Great Britain the incidence is 0.6 per million children (4, 12). Since 1998 data of children with JMML have been prospectively registered by the European Working Group of MDS and JMML in Childhood (EWOG-MDS), which has resulted in increased knowledge on the clinical presentation, the molecular heterogeneity, treatment efficacy and survival (Figure 6) (13).

Diagnostics:

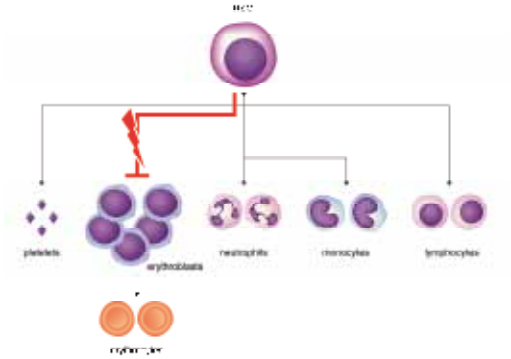
The diagnosis of JMML is mainly based on clinical presentation in combination with morphology of the peripheral blood smears and the molecular analysis of leukemic cells, which will be reviewed in chapter 2 (14).

Children with JMML present at young age (median age: 2 years) with pallor, malaise, fever, infections, skin infiltration, bleeding and coughing. Physical examination frequently reveals a (hepato)splenomegaly. Often juvenile xanthogranuloma are present (6, 11). Furthermore paraneoplastic phenomena such as rash, pleurisy, pericarditis, joint swelling and even hemophagocytosis are frequent presenting symptoms (15). There is also an association with Neurofibromatosis type 1 (NF1) and Noonan syndrome (1, 16).

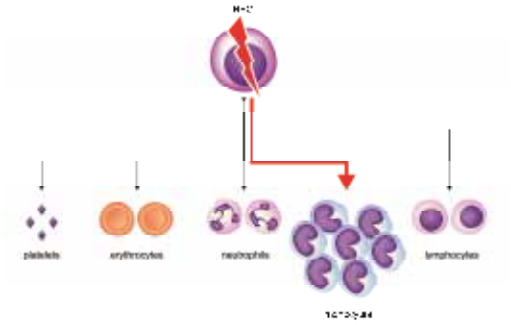
The typical presentation includes leukocytosis, anemia, thrombocytopenia, an absolute monocytosis (> 1 x 10^9/l) and a raised HbF. The blast percentage in the



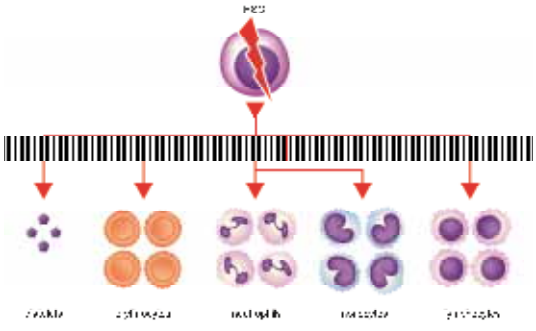
acute lymphoblastic leukemia



acute myeloid leukemia: acute erythroleukemia (M6)



juvenile myelomonocytic leukemia



myelodysplastic syndrome

Figure 5: Hematopoiesis in leukemia, JMML and MDS.

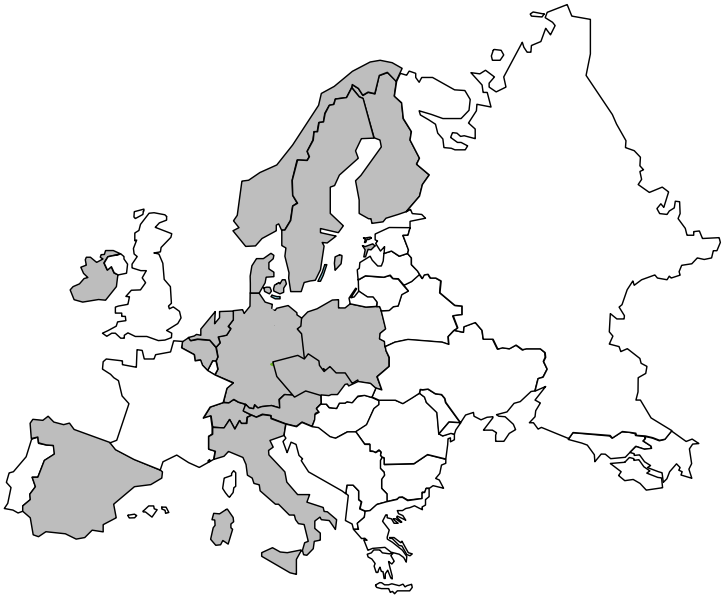


Figure 6: Countries participating in EWOG-MDS.

peripheral blood is less than 20% and myeloid precursors are found (14). Diagnosis is easier using the morphology of the peripheral blood than the bone marrow aspirate. The bone marrow is usually hyper-cellular with predominant granulocytic cells at all maturation stages and a blast percentage of less than 20%.

The diagnosis JMML requires the absence of Philadelphia chromosome or *BCR/ABL* rearrangement, as the presence of this is indicative for Chronic Myeloid Leukemia (CML). In 25% of patients with JMML, a monosomy 7 is found (Table 2) (14). Details regarding the clinical and molecular characteristics will be reviewed in chapter 2.

TREATMENT AND OUTCOME OF JMML

For JMML stem cell transplantation (SCT) is the only curative option. The disease is rapidly progressive, and the median survival without SCT is about a year (11). The 5-years disease-free survival after SCT with busulfan, cyclophosphamide and melphalan as conditioning regimen is around 50% (17). There is no difference in survival using a matched related donor or a matched unrelated donor (17). Graft-versus-leukemia may be an important therapeutic effect, illustrated by the fact that patients treated with an unrelated HSCT who developed chronic Graft Versus Host Disease had a lower risk of relapse and a better survival (18-19). The risk of recurrence within five years is 35% and most recurrences occur early. Risk factors for relapse include age > 4 years, female sex, increased HbF and a blast percentage above 20% (17). Mixed donor chimerism after SCT is predictive of relapse and a rapid re-transplantation may be indicated in such case. Infusions of donorlymphocytes have not proved to be useful (20-21).

Table 2: Diagnostic criteria Juvenile myelomonocytic leukemia

Juvenile myelomonocytic leukemie
1. clinical and hematological characteristics (all three mandatory)
Absolute monocytes in PB > 1 x 10^9/l
<5 % blasts in BM en PB < 20%
Splenomegaly*
2. oncogenetics (1 parameter necessary)
Somatic mutation in *PTPN11* of *RAS*, or *PTPN11*
germline *c-CBL*
NF 1 mutation or clinical diagnosis of NF1
Monosomy 7
3. in case no molecular aberration as mentioned at 2,
Absence of Philadelphia chromosome (BCR/ABL rearrangement)
+ two of the following criteria:
GM-CSF hypersensitivity
Raised HbF
Myeloïd precursors in PB
WBC in PB > 10 x 10^9/l
Clonal aberration other than monosomy 7

PB = peripheral blood
BM = bone marrow
NF1 = Neurofibromatosis type I
GM-CSF = granulocyte-macrophage colony stimulating factor
HbF = fetal hemoglobin
WBC = white blood cell count

Pre-transplantation treatment with chemotherapy is only useful to bridge the time till transplantation and the effect of this pre-transplantation chemotherapy is temporary and does not improve the final survival (22). No advantages of intensive chemotherapy or splenectomy prior to transplantation have been described (17).

CLINICAL CHARACTERISTICS, CLASSIFICATION AND DIAGNOSTICS OF MDS

Clinical characteristics:

The diagnosis of MDS is based on clinical symptoms, histo-morphological data and cytogenetic abnormalities. Patients with MDS often present with malaise, signs of anemia and/or thrombocytopenia and sometimes with recurrent infections associated with leukopenia. Hepatosplenomegaly is mainly found in advanced MDS (RAEB, RAEB-t). The diagnosis of MDS is confirmed by cytological and histological assessment of the bone

marrow aspirate, bone marrow biopsy and peripheral blood. These materials are evaluated for dysplastic features, the architecture of the bone marrow, the occurence of ring sideroblasts and myelofibrosis. The dysplasia is visible in at least two cell lines (7, 14, 23).

The annual incidence of MDS in children aged 0-14 years is 1.8 per million children (6). Since 1998 children with MDS are registered in the database of the European Working Group of MDS and JMML in Childhood (EWOG-MDS) (Figure 7). From this registry the following frequency of the different subtypes were found: 52% refractory cytopenia of childhood (RCC), 35% refractory anemia with excess of blasts (RAEB) and 13% refractory anemia with excess of blasts in transformation (RAEB-t) or myelodysplasia related-AML (MDR-AML) (14).

Classification:

In 1982 the first classification of MDS was made by the French American British group (FAB-classification). This morphological classification was based on the percentage of blasts in bone marrow and peripheral blood, the presence of ring sideroblasts in the bone marrow and the absolute monocyte count in the peripheral blood (24).

In 2001 a new WHO classification of MDS was published, in which besides the morphological features, cytogenetics were taken into account (Table 1A). For children a modification of the WHO classification became available (Table 1B) (6, 25). In 2008 an update of the WHO classification was published (7).

Refractory cytopenia of childhood (RCC): In the modified WHO classification for children RCC is used instead of refractory anemia in adults, as in children often a multilineage dysplasia is found in contrast to the anemia found in adults. In case of RCC less than 5% blasts are found in the peripheral blood and bone marrow and this type of MDS accounts for more than 50% of all MDS cases in childhood (26). The disease is difficult to differentiate from infections, metabolic disorders and vitamin deficiencies. Furthermore, diseases like Fanconi Anemia, Dyskeratosis Congenita, Paroxysmal Nocturnal Hemoglobinuria, Shwachman Diamond syndrome, amegakaryocytic thrombocytopenia and pancytopenia with radio-ulnair synostosis have to be excluded by physical examination or by molecular diagnostics. Furthermore, especially in children, RCC is characterized by a hypocellular bone marrow, which makes the differentiation from aplastic anemia and congenital bone marrow failure syndromes difficult. Apart from the clonal cytogenetic aberrations, at the moment there is no molecular marker to differentiate between aplastic anemia and MDS-RCC. Refractory anemia with ringed sideroblasts in childhood is rare. Myelofibrosis, an abnormal deposition of reticulin by fibroblasts in the bone marrow, is also less common in children as compared to adults and in children this myelofibrosis is mainly found in the 'advanced' MDS (RAEB or RAEB-t) patients (27).

'Advanced' MDS: When the blast percentage rises above 20% in children, this is called refractory anemia with excess of blasts in transformation (RAEB-t), and when the blast percentage is below the 20% it is called RAEB (6). When the blast percentage is increasing above the 30% the disease is referred to as myelodysplasia related acute myeloid leukemia (MDR-AML) (5-6, 25). However, when AML-specific cytogenetic aberrations are found, such as t(8;21)(q22;q22), t(15;17)(q22;q12) or inv(16)(p13q22), the disease is considered *de novo* AML irrespective of the blast count. In patients with

Down-syndrome, often dysplastic features are found in the bone marrow, but this is always considered leukemia of Down syndrome (ML-DS).

Secondary MDS: Apart from primary MDS, the disease can develop as secondary MDS after treatment with chemotherapy and / or radiotherapy, or after congenital bone marrow failure syndrome after aplastic anemia. In the past the group of patients who have a first degree relative with MDS (MDS family) were included in this group (6), but recently the EWOG-MDS decided to include the familial MDS cases in the primary MDS group, as they behave like primary MDS (7).

Genetics:

In approximately 50% of MDS patients an acquired clonal aberration is detected by conventional karyotyping and Fluorescent In Situ Hybrydisatie (FISH). The frequency varies in the different subtypes. In 75% of the primary RCC patients, in 33% of the advanced stages of MDS and in 20% of the secondary MDS cases a normal karyotype is found. Frequent cytogenetic abnormalities are monosomy 7 (10-20%), trisomy 8 (2-5%) and complex abnormalities, such as the prognostically relevant structural complex karyotype (2-35%) (28). RCC with monosomy 7 is associated with a rapid progression of disease (26). The relatively favorable cytogenetic abnormalities and Y-del (5q) frequently found in adults, are very rare in childhood (29).

Until recently no data were available about the role of molecular aberrations in the pathogenesis of childhood MDS. In this thesis we describe different studies about the role of molecular aberrations in childhood MDS.

TREATMENT AND OUTCOME OF CHILDHOOD MDS

The only curative option for MDS in childhood is myeloablative therapy followed by allogenic stem cell transplantation (SCT). In RCC a small group of patients show progression to leukemia, especially when monosomy 7 or complex karyotype is present (median time to progression less than two years), in contrast to patients with trisomy 8 and further karyotypes that show a more stable course of the disease (26). For children with RCC with monosomy 7 or complex karyotype, intensive conditioning with busulfan, melphalan and cyclophosphamide is recommended in an early stage of the disease (14). Currently, for the other RCC-patients a SCT with reduced intensity of the conditioning is advised when a HLA-matched donor is available (7/8 or 8/8 antigens) (30). In these cases, the conditioning consists of thiotepa, fludarabine, and anti-thymocyte globulin (14, 30). If no suitable donor is available, the patient is treated with optimal suppportive care.

For patients with RAEB and RAEB-t a SCT after conditioning with busulfan, melphalan and cyclophosphamide is needed as soon as possible (14). Patients with refractory cytopenia have a disease free survival of ~ 80% after transplantation with a matched related donor (31). For patients with RAEB and RAEB-t the disease-free survival after SCT is around 50% (31).

OUTLINE OF THIS THESIS

JMML and MDS are rare diseases in childhood. To date, myeloablative therapy followed by stem cell transplantation is the only curative treatment option. Research into the pathogenesis of both diseases will lead to better understanding of the diseases and this

may lead to new treatment options, in order to improve survival and reduce toxicity. In this thesis we aimed to identify new biological insights of the pathogenesis of JMML and MDS.

Part one (chapter 1-5) describes the studies we performed in JMML. In chapter 1 we review the new molecular insights and the mutational spectrum in JMML that has facilitated the diagnostic classification of JMML. Chapter 2 describes the role of *BRAF* mutations in the pathogenesis of JMML, whereas in chapter 3 we investigated the role of constitutively activated FLT3 in JMML. Chapter 4 describes the role of the *WT1* gene in JMML. In chapter 5 we present a JMML patient who received an HLA-identical umbilical cord blood transplantation from a sibling donor and a review of the literature about the use of this stem cell source as treatment modality in JMML.

Part two (chapter 6-9) describes the studies we performed in MDS. Chapter 6 is an analysis of the occurrence of the different type I and II genetic aberrations in 107 childhood MDS cases. In chapter 7 we describe the role of *IER3* expression in childhood MDS. In chapter 8 the role of mitochondrial DNA mutations in childhood MDS is analysed. In chapter 9 we report on the role of the immune system in the pathogenesis of childhood MDS. Chapter 10 summarizes and discusses the results and provides future perspectives.

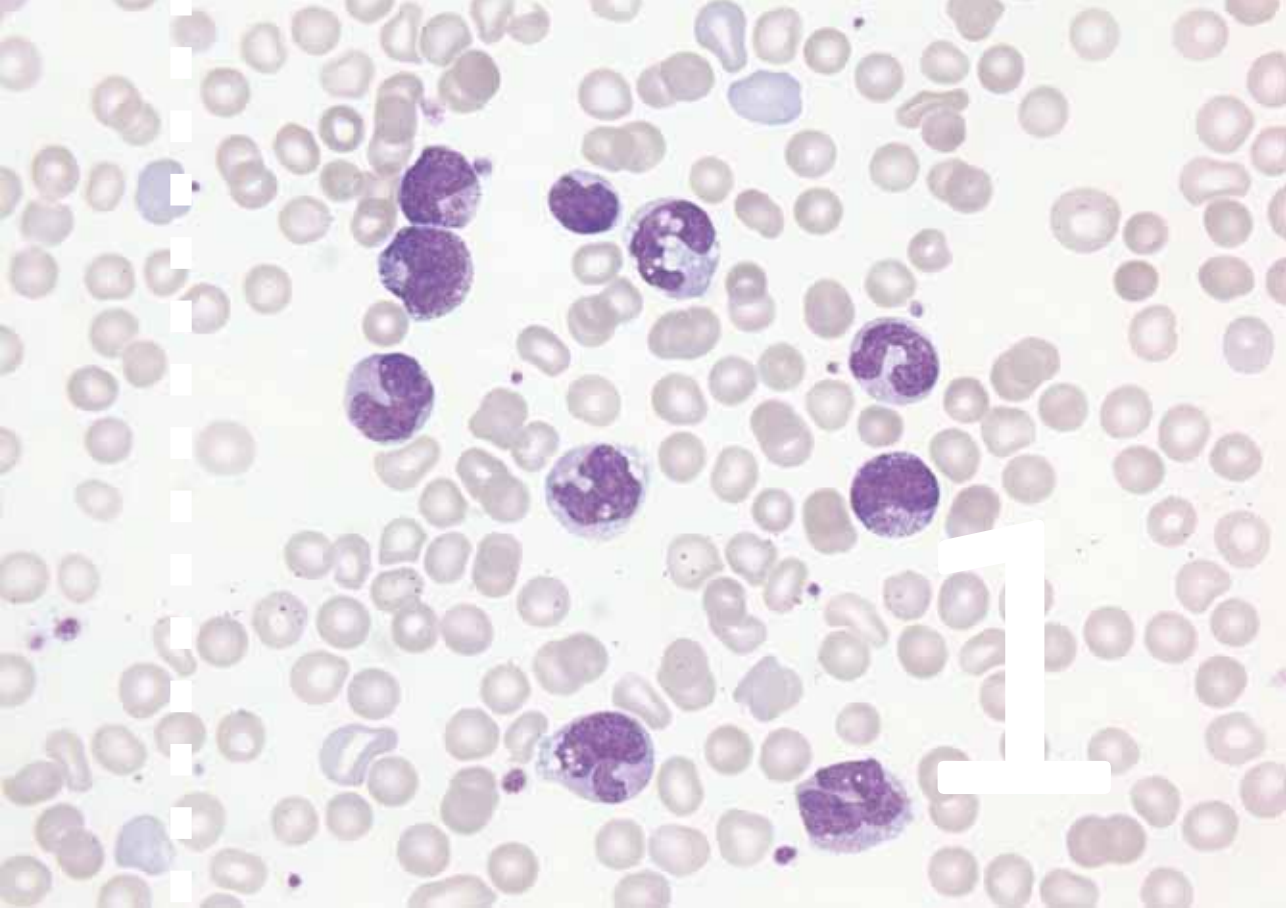
REFERENCES

1. Walter MJ, Ding L, Shen D, Shao J, Grillot M, McLellan M, et al. Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. Leukemia. 2011 Jul;25(7):1153-8.
2. de Vries AC, Zwaan CM, van den Heuvel-Eibrink MM. Molecular basis of juvenile myelomonocytic leukemia. Haematologica. 2010 Feb;95(2):179-82.
3. Dicker F, Haferlach C, Sundermann J, Wendland N, Weiss T, Kern W, et al. Mutation analysis for RUNX1, MLL-PTD, FLT3-ITD, NPM1 and NRAS in 269 patients with MDS or secondary AML. Leukemia. 2010 Aug;24(8):1528-32.
4. Passmore SJ, Chessells JM, Kempski H, Hann IM, Brownbill PA, Stiller CA. Paediatric myelodysplastic syndromes and juvenile myelomonocytic leukaemia in the UK: a population-based study of incidence and survival. Br J Haematol. 2003 Jun;121(5):758-67.
5. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. The World Health Organization classification of neoplasms of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting--Airlie House, Virginia, November, 1997. Hematol J. 2000;1(1):53-66.
6. Hasle H, Niemeyer CM, Chessells JM, Baumann I, Bennett JM, Kerndrup G, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia. 2003 Feb;17(2):277-82.
7. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. World Health Organization classification of tumours. Pathology and genetics of tumours of haematopoietic and lymphoid tissues: IARC Press, Lyon; 2008.
8. van Lom K, Houtsmuller AB, van Putten WL, Slater RM, Lowenberg B. Cytogenetic clonality analysis of megakaryocytes in myelodysplastic syndrome by dual-color fluorescence in situ hybridization and confocal laser scanning microscopy. Genes Chromosomes Cancer. 1999 Aug;25(4):332-8.
9. van Lom K, Hagemeijer A, Vandekerckhove F, Smit EM, Lowenberg B. Cytogenetic clonality analysis: typical patterns in myelodysplastic syndrome and acute myeloid leukaemia. Br J Haematol. 1996 Jun;93(3):594-600.
10. van Lom K, Hagemeijer A, Smit E, Hahlen K, Groeneveld K, Lowenberg B. Cytogenetic clonality analysis in myelodysplastic syndrome: monosomy 7 can be demonstrated in the myeloid and in the lymphoid lineage. Leukemia. 1995 Nov;9(11):1818-21.
11. Niemeyer CM, Arico M, Basso G, Biondi A, Cantu Rajnoldi A, Creutzig U, et al. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). Blood. 1997 May 15;89(10):3534-43.
12. Jadersten M, Saft L, Pellagatti A, Gohring G, Wainscoat JS, Boultwood J, et al. Clonal heterogeneity in the 5q- syndrome: p53 expressing progenitors prevail during lenalidomide treatment and expand at disease progression. Haematologica. 2009 Dec;94(12):1762-6.
13. Syndromes run together in the RAS pathway. Nat Genet. 2006 Mar;38(3):267.
14. EWOG-MDS. Clinical Trial Protocol EWOG-MDS 2006. Prospective non-randomized multi-center study for epidemiology and characterization of Myelodysplastic Syndromes (MDS) and Juvenile Myelomonocytic Leukemia (JMML) in childhood. 2007.

15. Gerritsen A, Lam K, Marion Schneider E, van den Heuvel-Eibrink MM. An exclusive case of juvenile myelomonocytic leukemia in association with Kikuchi's disease and hemophagocytic lymphohistiocytosis and a review of the literature. Leukemia research. 2006 Oct;30(10):1299-303.
16. Choong K, Freedman MH, Chitayat D, Kelly EN, Taylor G, Zipursky A. Juvenile myelomonocytic leukemia and Noonan syndrome. J Pediatr Hematol Oncol. 1999 Nov-Dec;21(6):523-7.
17. Locatelli F, Nollke P, Zecca M, Korthof E, Lanino E, Peters C, et al. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): results of the EWOG-MDS/EBMT trial. Blood. 2005 Jan 1;105(1):410-9.
18. Manabe A, Okamura J, Yumura-Yagi K, Akiyama Y, Sako M, Uchiyama H, et al. Allogeneic hematopoietic stem cell transplantation for 27 children with juvenile myelomonocytic leukemia diagnosed based on the criteria of the International JMML Working Group. Leukemia. 2002 Apr;16(4):645-9.
19. Smith FO, King R, Nelson G, Wagner JE, Robertson KA, Sanders JE, et al. Unrelated donor bone marrow transplantation for children with juvenile myelomonocytic leukaemia. Br J Haematol. 2002 Mar;116(3):716-24.
20. Yoshimi A, Niemeyer CM, Bohmer V, Duffner U, Strahm B, Kreyenberg H, et al. Chimaerism analyses and subsequent immunological intervention after stem cell transplantation in patients with juvenile myelomonocytic leukaemia. Br J Haematol. 2005 May;129(4):542-9.
21. Yoshimi A, Mohamed M, Bierings M, Urban C, Korthof E, Zecca M, et al. Second allogeneic hematopoietic stem cell transplantation (HSCT) results in outcome similar to that of first HSCT for patients with juvenile myelomonocytic leukemia. Leukemia. 2007 Mar;21(3):556-60.
22. Niemeyer CMea. AML-type intensive chemotherapy prior to stem cell transplantation does not improve survival in children and primary myelodysplastic syndromes. Blood. 2000;96:521a.
23. Cantu Rajnoldi A, Fenu S, Kerndrup G, van Wering ER, Niemeyer CM, Baumann I. Evaluation of dysplastic features in myelodysplastic syndromes: experience from the morphology group of the European Working Group of MDS in Childhood (EWOG-MDS). Annals of hematology. 2005 Jul;84(7):429-33.
24. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposals for the classification of the myelodysplastic syndromes. Br J Haematol. 1982 Jun;51(2):189-99.
25. Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. World Health Organization classification of tumours. Pathology and genetics. Tumours of Haematopoietic and Lymphoid tissues: IARC Press, Lyon; 2001.
26. Kardos G, Baumann I, Passmore SJ, Locatelli F, Hasle H, Schultz KR, et al. Refractory anemia in childhood: a retrospective analysis of 67 patients with particular reference to monosomy 7. Blood. 2003 Sep 15;102(6):1997-2003.
27. Maschek H, Georgii A, Kaloutsi V, Werner M, Bandecar K, Kressel MG, et al. Myelofibrosis in primary myelodysplastic syndromes: a retrospective study of 352 patients. European journal of haematology. 1992 Apr;48(4):208-14.
28. Gohring G, Michalova K, Beverloo HB, Betts D, Harbott J, Haas OA, et al. Complex karyotype newly defined: the strongest prognostic factor in advanced childhood myelodysplastic syndrome. Blood. 2010 Nov 11;116(19):3766-9.
29. Hasle H, Baumann I, Bergstrasser E, Fenu S, Fischer A, Kardos G, et al. The International Prognostic Scoring System (IPSS) for childhood myelodysplastic syndrome (MDS) and juvenile myelomonocytic leukemia (JMML). Leukemia. 2004 Dec;18(12):2008-14.
30. Rocquain J, Carbuccia N, Trouplin V, Raynaud S, Murati A, Nezri M, et al. Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. BMC Cancer. 2010;10:401.
31. EWOG-MDS. EWOG-MDS RC 06, TCRVbeta repertoire analysis and PNH clones in children with Refractory Cytopenia (RC). An open non-randomised multi-center prospective study. 2006.

PART ONE

JUVENILE MYELOMONOCYTIC LEUKEMIA



1

MOLECULAR BASIS OF JUVENILE MYELOMONOCYTIC LEUKEMIA

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in collaboration with EWOG-MDS

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Illustration: JMML, monocytosis and abnormal monocytes (PB)

INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) is classified as a combined myeloproliferative/myelodysplastic disease by the World Health Organization and accounts for less than 3% of all childhood haematological malignancies (1-2). Children typically present at young age (median age at diagnosis: 2 years) with hepatosplenomegaly, monocytosis, anemia, thrombocytopenia and elevated HbF (Figure 1) (3-4). Morphology of the peripheral blood smear is important for the diagnosis and shows low blast percentages and myeloid precursors. A bone marrow aspiration is necessary to exclude acute myelomonocytic leukemia, AML M4. In JMML, absence of the Philadelphia chromosome (*BCR/ABL* fusion gene) is one of the mandatory diagnostic criteria (3, 4). Cytogenetic analysis reveals monosomy 7 in 25% of the cases, random aberrations in the karyotype in 10%, whereas in 65% of the cases a normal karyotype is found (5). Figure 1 shows the currently used diagnostic criteria (3, 4).

MOLECULAR ABERRATIONS

1. RAS signalling pathway

In vitro granulocyte-macrophage colony stimulating factor (GM-CSF) hypersensitivity has been a hallmark of JMML for the past two decades (6). This GM-CSF hypersensitivity results from continuous activation of the GM-CSF-receptor-RAS-RAF-MEK-ERK signal transduction pathway. Currently, molecular aberrations, which will be discussed below,

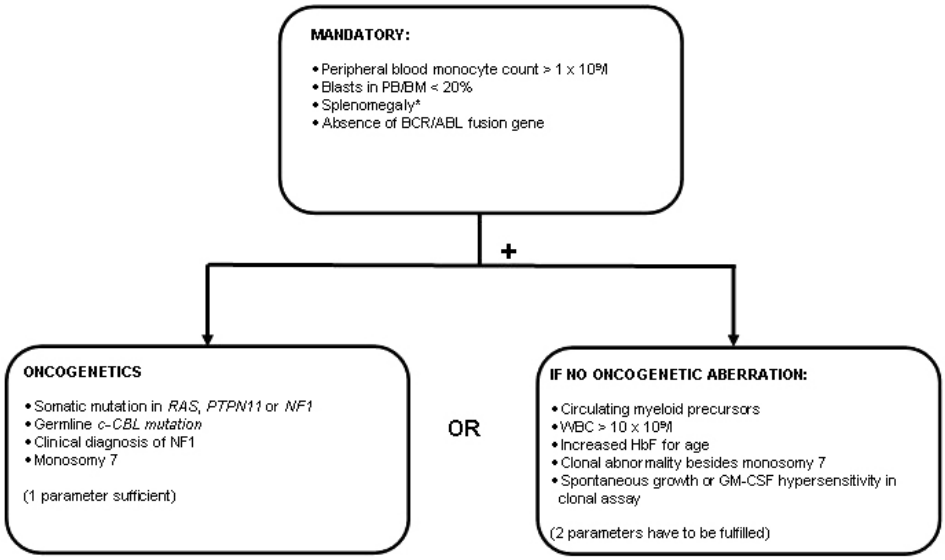


Figure 1: Diagnostic criteria of JMML. PB = peripheral blood; BM = bone marrow; NF1 = Neurofibromatosis type 1; WBC = white blood cell count; HbF = fetal hemoglobin; GM-CSF = granulocyte macrophage-colony stimulating factor; * Splenomegaly is present at first presentation in 95% of the cases. The majority of the patients is less than 13 years of age.

have become more important than the hypersensitivity assay for diagnostic classification (Figure 1) (3, 4).

Proteins encoded by the genes of the RAS family play a role in the transduction of extracellular signals to the nucleus and control proliferation and differentiation of many cell types (Figure 2). RAS proteins are signalling molecules, which regulate cellular processes by switching between an active (guanosine triphosphate (GTP) bound RAS) and an inactive (guanosine diphosphate (GDP) RAS) form. The active GTP RAS activates the RAF kinase, resulting in a downstream proliferative effect. The amount of GTP-RAS is regulated by guanine nucleotide exchange factors (GNEFs), and GTPase activating proteins (GAPs). GNEFs are necessary for the conversion of GDP-RAS into GTP-RAS, can be stimulated by upstream proteins like SHP1 and Son of Sevenless (SOS). GAPs, like neurofibromin, are responsible for the termination of the RAS signalling by RAS-GTP conversion in RAS-GDP (7). Somatic mutations in this pathway are frequently found in JMML, are mutually exclusive and result in continuously activation and cell proliferation. Analysis of genetic syndromes related to germline aberrations of the RAS pathway, like Neurofibromatosis type I (NF1) and Noonan syndrome, have increased the knowledge of the pathogenesis of JMML. In reverse, the discovery of somatic JMML related RAS-pathway aberrations has resulted in the discovery of germline RAS-pathway related mutations in Noonan like syndromes as Leopard syndrome, Costello syndrome and Cardio-Facio-Cutaneous syndrome (8-9).

NF1 mutations and clinical signs of Neurofibromatosis type 1:
Niemeyer *et al* reported that 11% of the JMML patients have clinical signs of Neurofibromatosis type 1 (5). Thereafter, Side *et al* found *NF1* gene mutations in 15% of the JMML patients without clinical signs of NF1 (Figure 2) (10). The *NF1* gene is a

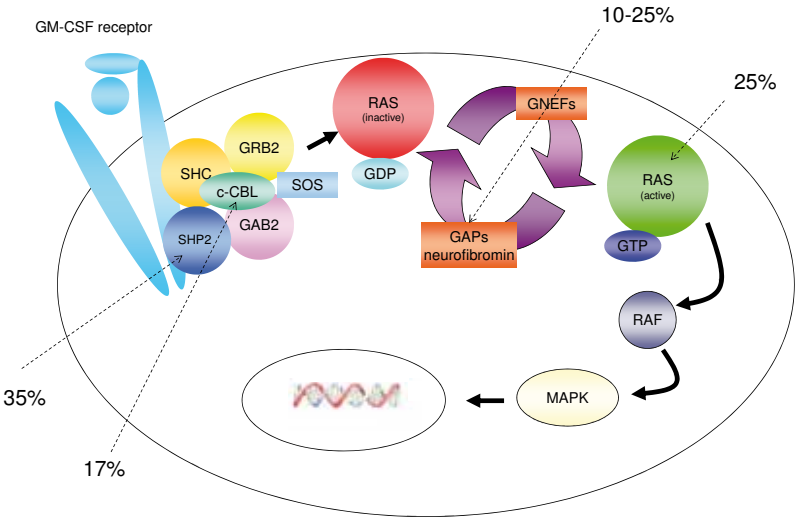


Figure 2: GM-CSF-receptor-RAS-RAF-MEK-ERK pathway. GNEF = guanine nucleotide exchange factor; GAP= GTPase activating proteins. In JMML in 35% of the cases a *PTPN11* mutation, in 20-25% of the cases a *RAS* mutation, in 17% a *c-CBL* mutation is found. In 10-25% of the cases clinical signs of NF1 or *NF1* gene mutations are found.

tumor suppressor gene encoding for neurofibromin, and is a GTPase activating protein hydrolysing GTP-RAS into GDP-RAS (10). JMML cells from children with NF1 showed a reduced neurofibromin activity, resulting in elevated GTP-RAS expression (11). In 2007, Flotho *et al* described somatic loss of heterozygosity (LOH) in 4 out of 5 JMML cases with NF1. In the leukemic cells the wild-type *NF1* gene was replaced by a second copy of the *NF1* mutant 17q arm, resulting from uniparental disomy (12). Steinemann *et al* described bi-allelic *NF1* gene inactivation in all cases in a larger cohort of JMML patients with NF1. Again, this was either caused by LOH (n=10), or by somatic mutations of the *NF1* gene (n=5) (13). This indicates that although NF1 predisposes for JMML and other leukemias, second events are necessary to abolish the complete function of the *NF1* gene in the development of malignancies. Recently, comparable bi-allelic mutations in the *NF1* gene were also found in non-syndromic AML and T-ALL patients, which underscores that bi-allelic inactivation of the *NF1* gene is an important mechanism in haematological malignancies, but also that it is not JMML-specific (14).

RAS gene mutations:
Mutations in the *RAS* genes are found in 20-30% of all human tumors. In 25% of all JMML patients, activating point mutations are found in codon 12, 13 and 61 of *NRAS* and *KRAS* resulting in a continuous activation of the RAS pathway (Figure 2) (15).

PTPN11 mutations:
PTPN11 is a gene encoding for the non-receptor protein tyrosine phosphatase SHP-2. Mutations in the *PTPN11* gene cause a gain of function of the SHP2 protein. This results in activation of the GNEFs and in this way to a continuous activation of RAS. Tartaglia *et al* observed somatic mutations in exon 3 and 13 of the *PTPN11* gene in 35% of the JMML patients without Noonan syndrome (Figure 2) (16).

Germline mutations in the *PTPN11* gene have been described in 50% of the Noonan syndrome cases. These mutations differ from the somatic mutations found in JMML (17). Noonan syndrome is characterized by developmental disorders, short stature, facial dysmorphia, skeletal anomalies and heart defects. Children with Noonan syndrome are at increased risk for developing JMML. However, in Noonan syndrome, JMML seems to behave differently from sporadic JMML as it occurs at a very young age (infancy) and tends to regress spontaneously (18-20). Therefore, recognising Noonan syndrome in a JMML patient is important, in order to identify those patients who might benefit from a watch and wait policy (17-18).

c-CBL mutations:
Recently Loh *et al* identified germline *c-CBL* mutations in 17% of the JMML patients, lacking *RAS*, *PTPN11* or *NF1* abnormalities (Figure 2). c-CBL is an E3 ubiquitin ligase, responsible for the intracellular transport and degradation of a large number of tyrosine kinase receptors, but has also important adaptor functions. One of the proteins regulated by c-CBL is Grb2. This adaptor molecule binds to c-CBL and in this way binding of c-CBL to SOS is prevented. Mutations in *c-CBL* have been shown to result in a continuous activation of RAS (21-22).

Other genes of the RAS pathway:
So up till now in about 80-85% of the JMML cases a somatic or germline mutation in the RAS pathway is found, indicating that hyperactivation of the RAS pathway plays a central

role in the pathogenesis of JMML. Therefore other genes involved in the RAS-RAF-MEK-ERK pathway have been investigated. Analysis of *SHC1, GRB2, GAB1, SOS1, BRAF* and *MEK 1* and *MEK 2* genes revealed no mutations (23-24). We and others showed that, upstream, FLT3 mutations are rare and found no constitutively activated FLT3 (25-26).

2. JAK-STAT pathway:
It has been suggested that JMML is the juvenile counterpart of chronic myelomonocytic leukemia (CMML) which mainly occurs in adults, as also in CMML GM-CSF hypersensitivity is found. However, *JAK2* mutations (V617F) which are found in in 3-13% of all CMML cases are a very rare event in JMML, underscoring the differences between JMML and CMML (26,27). Using flowcytometry Kotecha *et al* found hyperphosphorylation of pSTAT5 in response to subsaturating concentrations of GM-CSF. This supports the hypothesis that the JAK2-STAT5 pathway plays a collaborating role in the pathogenesis of JMML, but that JAK-STAT5 activation mainly seems to occur by upstream of activation of the RAS in the aberrant response of JMML cells to GM-CSF (21, 27).

3. PTEN:
Although all above mentioned studies have shown the role of hyperactivation of the RAS pathway in JMML, only few data are available documenting the status of the other components downstream of RAS, like P13K and MAPK. One of the other regulators of the P13K pathway is *PTEN*, a tumor suppressor gene which antagonizes the function of *P13K* and subsequently of *AKT*, and which is involved in cell growth, proliferation, apoptosis and differentiation. Liu *et al* found PTEN protein deficiency in 67% of the JMML patients and hypothesized that this deficiency might lead to insufficient negative growth signals to counter the hyperactive RAS pathway (28).

4. Epigenetics:
It has been suggested that PTEN protein deficiency can be caused by hypermethylation of the promoter region of *PTEN* (28). Apart from hypermethylation of *PTEN* it was recently shown that other epigenetic changes might play a role in the pathogenesis of JMML as illustrated by Furlan *et al* They reported the first JMML patient, also characterized by monosomy 7, treated with a DNA hypomethylating agent. The patient revealed an excellent clinical and molecular-genetic response, as illustrated by disappearance of the monosomy 7 and hypomethylation of the promoter region of the *CALCA* gene after treatment (29). In addition, other JMML studies showed mild hypermethylation of specific genes, like *p15, p16* and *RASSF1A* (30-31). These studies are important as they may point towards new, but already available, less toxic treatment directions in the near future for at least subsets of JMML patients.

TREATMENT AND PROGNOSIS

To date, the only curative treatment option for JMML is stem cell transplantation (SCT). Intensive chemotherapy does not represent a curative strategy, and splenectomy before transplantation has never shown to be of benefit (32). The median survival time without SCT is about 1 year (5). In the treatment protocol of the European Working Group of MDS and JMML in Childhood (EWOG-MDS) the 5 year event free survival after transplantation is in the 50% range and the cumulative relapse rate was 35%, with a therapy related

mortality rate of 15% (32). Results of a study performed by the Children's Oncology Group including a farnesyltransferase inhibitor in the pretransplantation window are underway (33). So far, no further data on treatment with RAS pathway inhibitors are available. As indicated above, hypomethylating agents may be a promising treatment option for the future.

Stem cell transplantation in this relatively young age category of children with JMML harbours the risk of serious toxicity, not only during the procedure but also in later life. Hence, there is a need for unravelling the biology of JMML in order to identify potential drugable targets.

CONCLUSION:

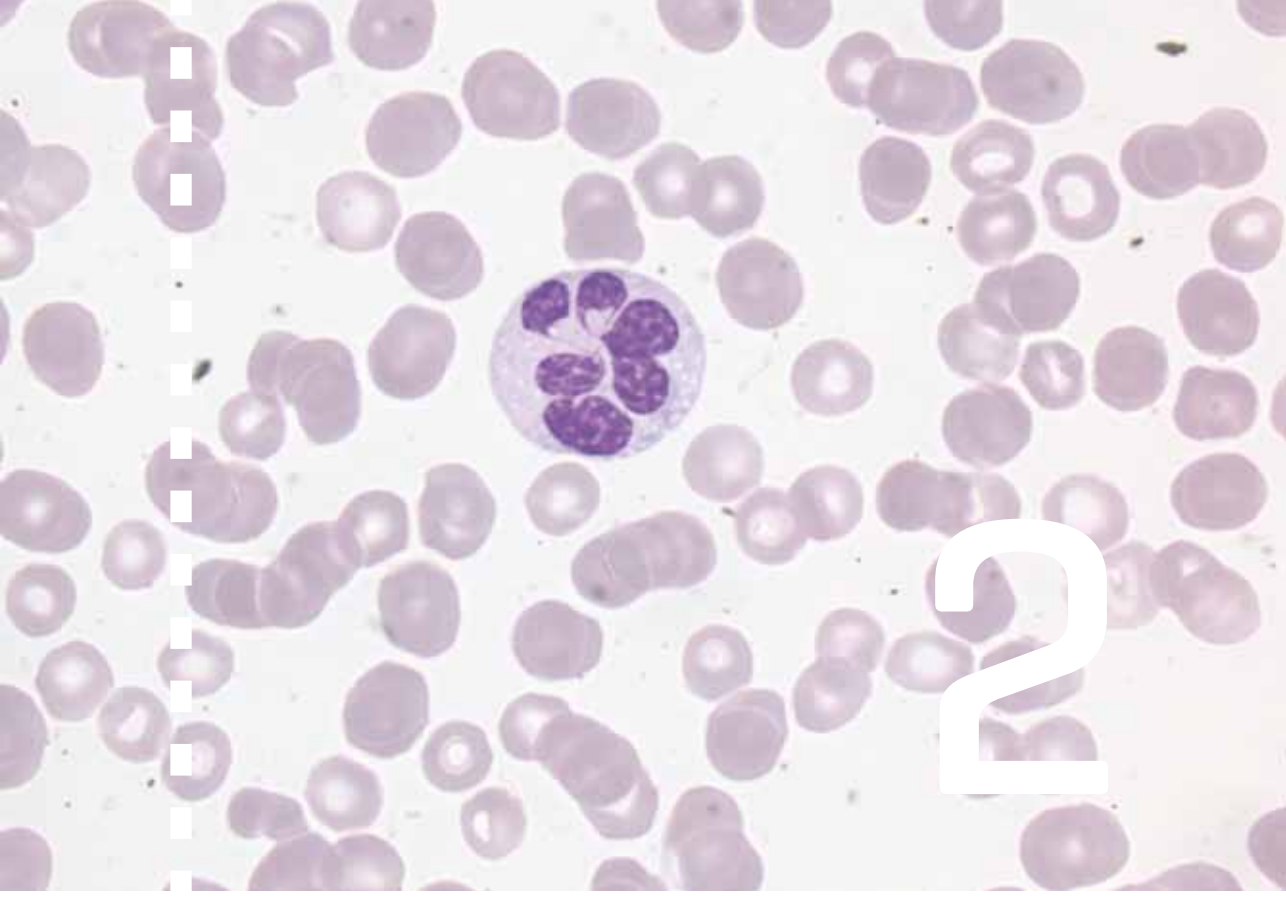
JMML is a rare hematological malignancy in childhood. Increased knowledge of the molecular background has enhanced diagnostic classification as in more than 80% of the cases mutations in RAS pathway related genes can be found.

REFERENCES

1. Hasle H, Wadsworth LD, Massing BG, McBride M, Schultz KR. A population-based study of childhood myelodysplastic syndrome in British Columbia, Canada. Br J Haematol. 1999 Sep;106(4):1027-1032.
2. Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. World Health Organization classification of tumours. Pathology and genetics. Tumours of Haematopoietic and Lymphoid tissues: IARC Press, Lyon; 2001.
3. EWOG-MDS. Clinical Trial Protocol EWOG-MDS 2006. Prospective non-randomized multi-center study for epidemiology and characterization of Myelodysplastic Syndromes (MDS) and Juvenile Myelomonocytic Leukemia (JMML) in childhood. 2007.
4. Chan RJ, Cooper T, Kratz CP, Weiss B, Loh ML. Juvenile myelomonocytic leukemia: a report from the 2nd International JMML Symposium. Leukemia research. 2009 Mar;33(3):355-362.
5. Niemeyer CM, Arico M, Basso G, Biondi A, Cantu Rajnoldi A, Creutzig U, Haas O, Harbott J, Hasle H, Kerndrup G, Locatelli F, Mann G, Stollmann-Gibbels B, van't Veer-Korthof ET, van Wering E, Zimmermann M. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). Blood. 1997 May 15;89(10):3534-3543.
6. Emanuel PD, Bates LJ, Castleberry RP, Gualtieri RJ, Zuckerman KS. Selective hypersensitivity to granulocyte-macrophage colony-stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors. Blood. 1991 Mar 1;77(5):925-929.
7. Emanuel PD. RAS pathway mutations in juvenile myelomonocytic leukemia. Acta haematologica. 2008;119(4):207-211.
8. Kratz CP, Schubbert S, Bollag G, Niemeyer CM, Shannon KM, Zenker M. Germline mutations in components of the Ras signaling pathway in Noonan syndrome and related disorders. Cell Cycle. 2006 Aug;5(15):1607-1611.
9. Syndromes run together in the RAS pathway. Nat Genet. 2006 Mar;38(3):267.
10. Side LE, Emanuel PD, Taylor B, Franklin J, Thompson P, Castleberry RP, Shannon KM. Mutations of the NF1 gene in children with juvenile myelomonocytic leukemia without clinical evidence of neurofibromatosis, type 1. Blood. 1998 Jul 1;92(1):267-272.
11. Bollag G, Clapp DW, Shih S, Adler F, Zhang YY, Thompson P, Lange BJ, Freedman MH, McCormick F, Jacks T, Shannon K. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. Nat Genet. 1996 Feb;12(2):144-148.
12. Flotho C, Steinemann D, Mullighan CG, Neale G, Mayer K, Kratz CP, Schlegelberger B, Downing JR, Niemeyer CM. Genome-wide single-nucleotide polymorphism analysis in juvenile myelomonocytic leukemia identifies uniparental disomy surrounding the NF1 locus in cases associated with neurofibromatosis but not in cases with mutant RAS or PTPN11. Oncogene. 2007 Aug 23;26(39):5816-5821.
13. Steinemann D, Arning L, Praulich I, Stuhrmann M, Hasle H, Stary J, Schlegelberger B, Niemeyer CM, Flotho C. Mitotic recombination and compound-heterozygous mutations are predominant NF1-inactivating mechanisms in children with juvenile myelomonocytic leukemia and neurofibromatosis type 1. Haematologica. 2010 Feb;95(2):320-323.
14. Balgobind BV, Van Vlierberghe P, van den Ouweland AM, Beverloo HB, Terlouw-Kromosoeto JN, van Wering ER, Reinhardt D, Horstmann M, Kaspers GJ, Pieters R, Zwaan CM, Van den Heuvel-Eibrink MM,

Meijerink JP. Leukemia-associated NF1 inactivation in patients with pediatric T-ALL and AML lacking evidence for neurofibromatosis. Blood. 2008 Apr 15;111(8):4322-4328.

15. Flotho C, Valcamonica S, Mach-Pascual S, Schmahl G, Corral L, Ritterbach J, Hasle H, Arico M, Biondi A, Niemeyer CM. RAS mutations and clonality analysis in children with juvenile myelomonocytic leukemia (JMML). Leukemia. 1999 Jan;13(1):32-37.
16. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J, Jung A, Hahlen K, Hasle H, Licht JD, Gelb BD. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nature genetics. 2003 Jun;34(2):148-150.
17. Kratz CP, Niemeyer CM, Castleberry RP, Cetin M, Bergstrasser E, Emanuel PD, Hasle H, Kardos G, Klein C, Kojima S, Stary J, Trebo M, Zecca M, Gelb BD, Tartaglia M, Loh ML. The mutational spectrum of PTPN11 in juvenile myelomonocytic leukemia and Noonan syndrome/myeloproliferative disease. Blood. 2005 Sep 15;106(6):2183-2185.
18. Bader-Meunier B, Tchernia G, Mielot F, Fontaine JL, Thomas C, Lyonnet S, Lavergne JM, Dommergues JP. Occurrence of myeloproliferative disorder in patients with Noonan syndrome. The Journal of pediatrics. 1997 Jun;130(6):885-889.
19. Fukuda M, Horibe K, Miyajima Y, Matsumoto K, Nagashima M. Spontaneous remission of juvenile chronic myelomonocytic leukemia in an infant with Noonan syndrome. J Pediatr Hematol Oncol. 1997 Mar-Apr;19(2):177-179.
20. Silvio F, Carlo L, Elena B, Nicoletta B, Daniela F, Roberto M. Transient abnormal myelopoiesis in Noonan syndrome. J Pediatr Hematol Oncol. 2002 Dec;24(9):763-764.
21. Loh ML, Sakai DS, Flotho C, Kang M, Fliegauf M, Archambeault S, Mullighan CG, Chen L, Bergstraesser E, Bueso-Ramos CE, Emanuel PD, Hasle H, Issa JP, van den Heuvel-Eibrink MM, Locatelli F, Stary J, Trebo M, Wlodarski M, Zecca M, Shannon KM, Niemeyer CM. Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. Blood. 2009 Aug 27;114(9):1859-1863.
22. Schmidt MH, Dikic I. The Cbl interactome and its functions. Nature reviews. 2005 Dec;6(12):907-918.
23. de Vries AC, Stam RW, Kratz CP, Zenker M, Niemeyer CM, van den Heuvel-Eibrink MM. Mutation analysis of the BRAF oncogene in juvenile myelomonocytic leukemia. Haematologica. 2007 Nov;92(11):1574-1575.
24. Kratz CP, Niemeyer CM, Thomas C, Bauhuber S, Matejas V, Bergstrasser E, Flotho C, Flores NJ, Haas O, Hasle H, van den Heuvel-Eibrink MM, Kucherlapati RS, Lang P, Roberts AE, Stary J, Strahm B, Swanson KD, Trebo M, Zecca M, Neel B, Locatelli F, Loh ML, Zenker M. Mutation analysis of Son of Sevenless in juvenile myelomonocytic leukemia. Leukemia. 2007 May;21(5):1108-1109.
25. de Vries AC, Stam RW, Schneider P, Niemeyer CM, van Wering ER, Haas OA, Kratz CP, den Boer ML, Pieters R, van den Heuvel-Eibrink MM. Role of mutation independent constitutive activation of FLT3 in juvenile myelomonocytic leukemia. Haematologica. 2007 Nov;92(11):1557-1560.
26. Gratias EJ, Liu YL, Meleth S, Castleberry RP, Emanuel PD. Activating FLT3 mutations are rare in children with juvenile myelomonocytic leukemia. Pediatr Blood Cancer. 2005 Feb;44(2):142-146.
27. Kotecha N, Flores NJ, Irish JM, Simonds EF, Sakai DS, Archambeault S, Diaz-Flores E, Coram M, Shannon KM, Nolan GP, Loh ML. Single-cell profiling identifies aberrant STAT5 activation in myeloid malignancies with specific clinical and biologic correlates. Cancer Cell. 2008 Oct 7;14(4):335-343.
28. Liu YL, Castleberry RP, Emanuel PD. PTEN deficiency is a common defect in juvenile myelomonocytic leukemia. Leukemia research. 2009 May;33(5):671-677.
29. Furlan I, Batz C, Flotho C, Mohr B, Lubbert M, Suttorp M, Niemeyer CM. Intriguing response to azacitidine in a patient with juvenile myelomonocytic leukemia and monosomy 7. Blood. 2009 Mar 19;113(12):2867-2868.
30. Hasegawa D, Manabe A, Kubota T, Kawasaki H, Hirose I, Ohtsuka Y, Tsuruta T, Ebihara Y, Goto Y, Zhao XY, Sakashita K, Koike K, Isomura M, Kojima S, Hoshika A, Tsuji K, Nakahata T. Methylation status of the p15 and p16 genes in paediatric myelodysplastic syndrome and juvenile myelomonocytic leukaemia. Br J Haematol. 2005 Mar;128(6):805-812.
31. Johan MF, Bowen DT, Frew ME, Goodeve AC, Reilly JT. Aberrant methylation of the negative regulators RASSFIA, SHP-1 and SOCS-1 in myelodysplastic syndromes and acute myeloid leukaemia. Br J Haematol. 2005 Apr;129(1):60-65.
32. Locatelli F, Nollke P, Zecca M, Korthof E, Lanino E, Peters C, Pession A, Kabisch H, Uderzo C, Bonfim CS, Bader P, Dilloo D, Stary J, Fischer A, Revesz T, Fuhrer M, Hasle H, Trebo M, van den Heuvel-Eibrink MM, Fenu S, Strahm B, Giorgiani G, Bonora MR, Duffner U, Niemeyer CM. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): results of the EWOG-MDS/EBMT trial. Blood. 2005 Jan 1;105(1):410-419.
33. EWOG-MDS. EWOG-MDS RC 06, TCRVbeta repertoire analysis and PNH clones in children with Refractory Cytopenia (RC). An open non-randomised multi-center prospective study. 2006.



2

BRAF MUTATIONS IN JUVENILE MYELOMONOCYTIC LEUKEMIA

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(EWOG-MDS)

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illustration: JMML, abnormal neutrophil (PB)

ABSTRACT

Patients with juvenile myelomonocytic leukemia (JMML) harbour somatic mutations in *PTPN11* and *RAS* genes or have clinical signs of Neurofibromatosis. The remaining cases presumably carry somatic mutations in other genes in the RAS pathway. BRAF plays a central role in this pathway between RAS and downstream molecules including MEK and ERK. *BRAF* mutations frequently occur in cancer. Recently, *BRAF* mutations were found in leukemia. Besides that, germline *BRAF* mutations cause cardio-facio-cutaneous syndrome, which shares many features with Noonan syndrome, which predisposes to a myeloproliferative disease resembling JMML

In 65 JMML patients screening for V600E mutations in exon 15 was performed. In a subset of patients, without *RAS* or *PTPN11* mutations and no clinical signs of Neurofibromatosis type 1, the entire coding sequence of *BRAF* was analyzed. Sequence analysis was performed by direct, bidirectional sequencing of purified polymerase chain reaction products.

Since we did not identify any *BRAF* mutation, we conclude that this gene is unlikely to play a role in the pathogenesis of JMML.

INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) is a rare malignant disease in children, accounting for less than 3% of all childhood hematological malignancies. The disease, mainly affecting young children, is characterized by prominent hepatosplenomegaly, absolute monocytosis, presence of myeloid precursors in peripheral blood, low platelet count, frequent skin involvement and *in vitro* granulocyte-macrophage colony stimulating factor (GM-CSF) hypersensitivity (1). GM-CSF hypersensitivity in JMML results from continuous activation of the GM-CSF-receptor-RAS-RAF-MEK-ERK signal transduction pathway due to activating mutations of *RAS* (2) or *PTPN11* (3-5). These mutations occur in 25% and 35% of JMML cases, respectively. Moreover, approximately 11% of the JMML cases are associated with clinical Neurofibromatosis type 1 (NF1) and JMML cells show bi-allelic inactivation of the *NF1* tumor suppressor gene encoding for neurofibromin, a GTPase activating protein for Ras. Loss of *NF1* leads to activation of RAS signaling (6, 7). The remaining cases presumably carry somatic mutations in other genes of the RAS signaling pathway.

The RAS effector BRAF plays a central role in transmitting growth signals from RAS to downstream molecules including MEK and ERK. Somatic mutations in *BRAF* occur at high frequency in numerous human cancers (8, 9). The *BRAF* V600E mutation results in increased kinase activity and accounts for more than 90% of the mutations (8, 10). Germline mutations in *BRAF* cause cardio-facio-cutaneous syndrome (CFC), a dominant disorder characterized by short stature, cardiac defects, distinct facial features, developmental delay, and ectodermal abnormalities (11, 12). CFC syndrome shares many features with other dominant syndromes including Costello and Noonan syndrome (NS) (13). NS is caused by germline mutations in genes of the RAS pathway, including *PTPN11*, *KRAS*, or *SOS1* (14). Interestingly, infants with NS are predisposed to develop a myeloproliferative disease resembling JMML (3). So far, to our knowledge, studies on downstream *BRAF* mutations have not been reported in JMML. In this paper we studied the role of *BRAF* mutations in JMML patients.

PATIENTS AND METHODS

Patients

In this study, 65 JMML patients were included. Informed consent from the parents was obtained according to the Helsinki declaration. The diagnosis of JMML was based on the criteria described by Niemeyer *et al* (15). Patients were treated according to the guidelines for JMML of the European Working Group of MDS and JMML in Childhood (EWOG- MDS).

Methods

Screening for *BRAF* mutations was performed from mononuclear cells derived from cryopreserved primary bone marrow, peripheral blood or spleen (16) at diagnosis. Cells were thawed and resuspended in RMPI 1640 medium (Dutch modification without L-glutamine; 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, 0,125 µg/ml fungizone and 0,2 mg/ml gentamycin (Invitrogen).

Genomic DNA was extracted from a minimum of 5 x 10^6 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantification of DNA was performed using a spectrophotometer. Sequence analysis was performed using forward and reverse primers for *BRAF* exon 15: forward 5'- AGC CCC AAA AAT CTT AAA AG- 3' and reverse 5'- CTC AGG GCC AAA AAT TTA AT- 3'. Screening for V600E mutation in exon 15 was carried out by direct, bidirectional sequencing of purified polymerase chain reaction (PCR) products using the BigDye Terminator v1.1 cycle sequencing protocol (Applied Biosystems, Foster City, CA, USA) and an ABI3100 Genetic Analyzer (Applied Biosystems). In a subset of patients the entire coding sequence of *BRAF* was analyzed. These samples were tested by direct, bidirectional sequencing of purified polymerase chain reaction (PCR) products using the ABI BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, California) and an ABI3730 Capillary Array Sequencer (Applied Biosystems). Details on primers are available on request.

RESULTS AND DISCUSSION

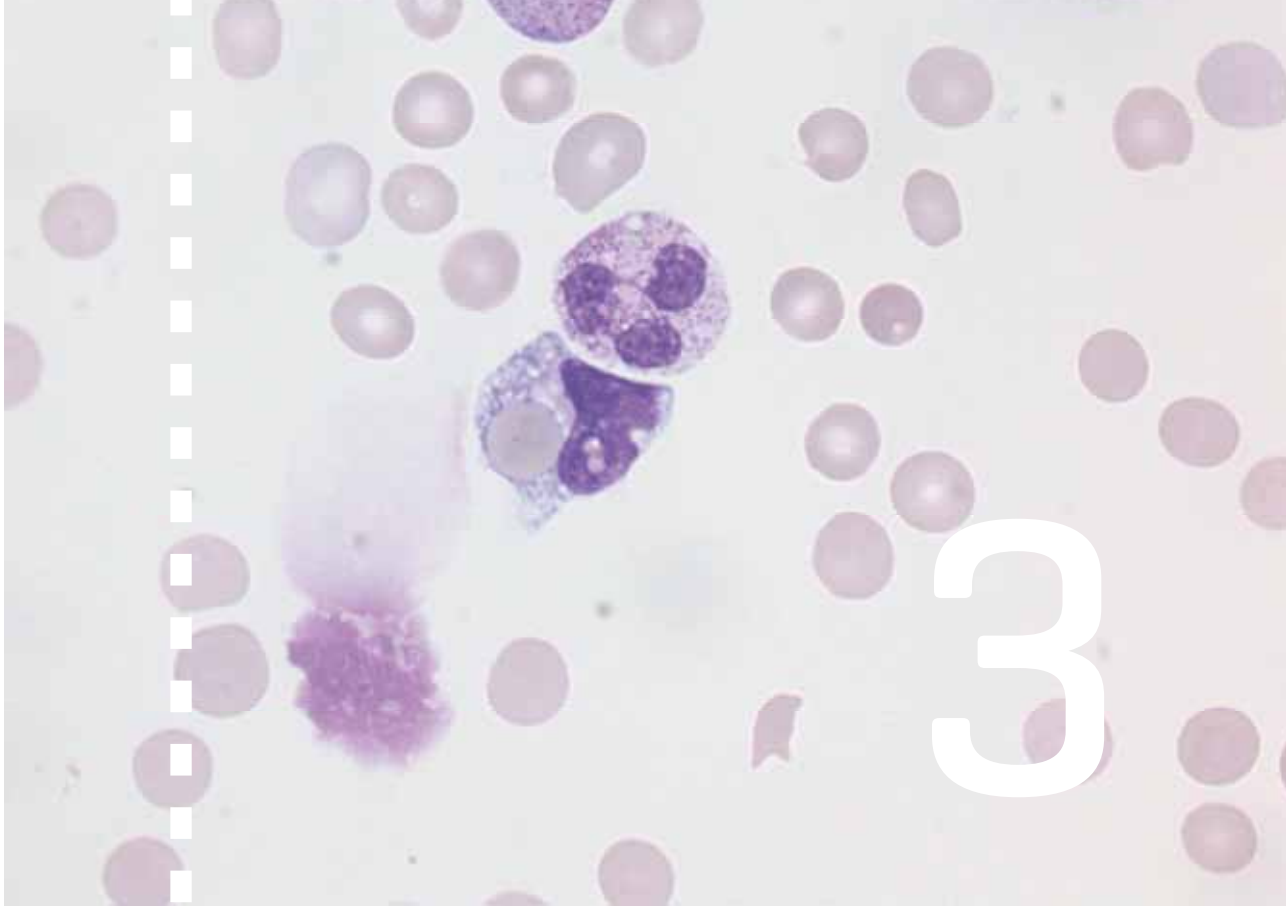
Of the 65 patients with JMML included in this study, 26 had no clinical diagnosis of NF1 and lacked mutations of *RAS, PTPN11* of *NF1*. Six patients had a clinical diagnosis of NF1, 12 had a somatic mutation of *NRAS* or *KRAS* and 12 patients had a somatic mutation of *PTPN11*. No data on the molecular background were available for nine patients. In none of the 65 patients a V600E mutation of the *BRAF* gene was found. Moreover, in a subset of 15 patients without a clinical diagnosis of NF1 and without mutations of *PTPN11* or *RAS,* the entire coding region of *BRAF* was analyzed. In this elected group of patients no mutations were identified.

JMML is regarded as a model disease that is due to hyperactive RAS signaling. Mutant proteins of the RAS-RAF-MEK-ERK pathway play an important role in the pathogenesis of JMML and explain the GM-CSF hypersensitivity observed *ex vivo*. In the majority of cases of JMML, these mutations are mutually exclusive and affect *NRAS, KRAS, NF1,* or *PTPN11* genes. Additionally, mutations in this pathway function as an important diagnostic tool and are a potential marker of minimal residual disease (17) and a potential therapeutic target in future treatment protocols.

Our hypothesis was that BRAF might play an important role in JMML as it is an important downstream effector of RAS. Somatic *BRAF* mutations occur frequently in other types of human cancer and recently, *BRAF* mutations were found in leukemia. To our knowledge, this is the first study that evaluated the occurrence of *BRAF* mutations in JMML. Our data show that *BRAF* mutations do not play a major role in JMML. Therefore, additional analysis of genes of the RAS pathway will be necessary to identify genetic aberrations in cases without known mutations.

REFERENCES

1. Emanuel PD, Bates LJ, Castleberry RP, Gualtieri RJ, Zuckerman KS. Selective hypersensitivity to granulocyte-macrophage colony-stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors. Blood. 1991 Mar 1;77(5):925-929.
2. Kalra R, Paderanga DC, Olson K, Shannon KM. Genetic analysis is consistent with the hypothesis that NF1 limits myeloid cell growth through p21ras. Blood. 1994 Nov 15;84(10):3435-3439.
3. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J, Jung A, Hahlen K, Hasle H, Licht JD, Gelb BD. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat Genet. 2003 Jun;34(2):148-150.
4. Kratz CP, Niemeyer CM, Castleberry RP, Cetin M, Bergstrasser E, Emanuel PD, Hasle H, Kardos G, Klein C, Kojima S, Stary J, Trebo M, Zecca M, Gelb BD, Tartaglia M, Loh ML. The mutational spectrum of PTPN11 in juvenile myelomonocytic leukemia and Noonan syndrome/myeloproliferative disease. Blood. 2005 Sep 15;106(6):2183-2185.
5. Loh ML, Vattikuti S, Schubbert S, Reynolds MG, Carlson E, Lieuw KH, Cheng JW, Lee CM, Stokoe D, Bonifas JM, Curtiss NP, Gotlib J, Meshinchi S, Le Beau MM, Emanuel PD, Shannon KM. Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis. Blood. 2004 Mar 15;103(6):2325-2331.
6. Side LE, Emanuel PD, Taylor B, Franklin J, Thompson P, Castleberry RP, Shannon KM. Mutations of the NF1 gene in children with juvenile myelomonocytic leukemia without clinical evidence of neurofibromatosis, type 1. Blood. 1998 Jul 1;92(1):267-272.
7. Bollag G, Clapp DW, Shih S, Adler F, Zhang YY, Thompson P, Lange BJ, Freedman MH, McCormick F, Jacks T, Shannon K. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. Nat Genet. 1996 Feb;12(2):144-148.
8. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. Nature. 2002 Jun 27;417(6892):949-954.
9. Tuveson DA, Weber BL, Herlyn M. BRAF as a potential therapeutic target in melanoma and other malignancies. Cancer Cell. 2003 Aug;4(2):95-98.
10. Sridhar SS, Hedley D, Siu LL. Raf kinase as a target for anticancer therapeutics. Mol Cancer Ther. 2005 Apr;4(4):677-685.
11. Niihori T, Aoki Y, Narumi Y, Neri G, Cave H, Verloes A, Okamoto N, Hennekam RC, Gillessen-Kaesbach G, Wieczorek D, Kavamura MI, Kurosawa K, Ohashi H, Wilson L, Heron D, Bonneau D, Corona G, Kaname T, Naritomi K, Baumann C, Matsumoto N, Kato K, Kure S, Matsubara Y. Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. Nat Genet. 2006 Mar;38(3):294-296.
12. Rodriguez-Viciana P, Tetsu O, Tidyman WE, Estep AL, Conger BA, Santa Cruz M, McCormick F, Rauen K. Germline mutations in genes within the MAPK pathway cause cardio-facio-cutaneous syndrome. Science. 2006;311(5765):1287-1290
13. Kratz CP, Niemeyer CM, Zenker M. An unexpected new role of mutant Ras: perturbation of human embryonic development. J Mol Med. 2007 Jan 9.
14. Gelb BD, Tartaglia M. Noonan syndrome and related disorders: dysregulated RAS-mitogen activated protein kinase signal transduction. Hum Mol Genet. 2006 Oct 15;15 Spec No 2:R220-226.
15. Niemeyer CM, Arico M, Basso G, Biondi A, Cantu Rajnoldi A, Creutzig U, Haas O, Harbott J, Hasle H, Kerndrup G, Locatelli F, Mann G, Stollmann-Gibbels B, van't Veer-Korthof ET, van Wering E, Zimmermann M. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). Blood. 1997 May 15;89(10):3534-3543.
16. Gerhardt TM, Schmahl GE, Flotho C, Rath AV, Niemeyer CM. Expression of the Evi-1 gene in haemopoietic cells of children with juvenile myelomonocytic leukaemia and normal donors. Br J Haematol. 1997 Dec;99(4):882-887.
17. Archambeault S, Yoshimi A, Kratz CP, Reising M, Fischer A, Noellke P, Locatelli F, Sedlacek C, Flotho C, Zecca M, Emanuel PD, Castleberry RP, Niemeyer CM, Bader P, Loh ML. Development of an allele-specific minimal residual disease assay for patients with Juvenile Myelomonocytic Leukemia-moving beyond clinical assessment. Blood. 2006 November 16, 2006;108(11).



3

ROLE OF MUTATION INDEPENDENT CONSTITUTIVE ACTIVATION OF FLT3 IN JUVENILE MYELOMONOCYTIC LEUKEMIA

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Illustration: JMML, hemophagocytosis (PB)

ABSTRACT

Background and objective: Activating *FLT3* mutations have been identified as prognostic factors in several myeloid malignancies. In addition and independent of these mutations, ligand-independent activation of FLT3 by wild-type overexpression as well as ligand-dependent activation in leukemic cells co-expressing FLT3 ligand (FLT3 ligand) resulting in autocrine FLT3 signaling have been described in leukemia. Although in juvenile myelomonocytic leukemia (JMML) *FLT3* internal tandem duplications (*FLT3/ITDs*) and mutations affecting the tyrosine kinase domain (*TKD*) are rare, no data are available on expression levels of *FLT3* and *FLT3 ligand* in JMML.

Design and methods: Fifty-one JMML patients were screened for the presence of activating *FLT3/ITDs* and *FLT3/TKD* mutations. In 21 of these patients *FLT3* and *FLT3 ligand* mRNA levels were assessed using real-time quantitative PCR (Taqman). MTT assays were performed to assess the sensitivity of JMML cells to the FLT3 inhibitor PKC412.

Results: In none of the 51 JMML samples *FLT3/ITDs* or *TDK* mutations were found. *FLT3* appeared to be expressed at higher levels as compared to healthy controls, but no PKC412 cytotoxicity was found in JMML, this in contrast to *MLL*-rearranged ALL infants with activated FLT3. Moreover, no evidence of ligand independent FLT3 activation was found.

Conclusion: We conclude that constitutively activated FLT3 does not occur in JMML and that treatment with FLT3 inhibitors is unlikely to be effective in JMML.

INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) is a rare malignant disease in children, accounting for less than 3% of all childhood hematologic malignancies. The disease is characterized by young age, prominent hepatosplenomegaly, the presence of myeloid precursors in the peripheral blood smears, low platelet count, frequent skin involvement and *in vitro* granulocyte-macrophage colony stimulating factor (GM-CSF) hypersensitivity. JMML is associated with the monosomy 7 karyotype in about 25% of the cases and with Neurofibromatosis type 1. GM-CSF hypersensitivity in JMML results from continuous activation of the GM-CSF-receptor-RAS signal transduction pathway caused by activating *RAS* mutations (1), somatic *PTPN11* mutations (2) or loss of heterozygosity of *NF1* (3) occuring mutually exclusive in 25%, 35% and 10-15% of the JMML cases respectively.

Therefore it may be plausible that a proportion of JMML patients carry activating mutations in *FLT3*, like in AML where *RAS, PTPN11* and *FLT3* gene mutations occur mutually exclusive (4-13). FLT3 is a member of the class III receptor tyrosine kinase (RTK) family, which receptors are involved in proliferation and differentiation of hematopoietic cells (5, 14). FLT3 activation is induced upon binding of its ligand (FLT3 ligand) resulting in activation of the downstream signal transduction pathway promoting survival and proliferation. Alternatively, ligand independent activation has been described in leukemia either by overexpression of the wild-type FLT3 receptor or by activating mutations in the *FLT3* gene, like internal tandem duplications (ITDs) within the juxtamembrane (JM) region or point-mutations in the tyrosine kinase domain (TDK) (15-17).

In JMML until now, apart from allogenic stem cell transplantation (allo SCT) no curative treatment is available and only about 50% of all patients eventually survive (18). Recently, several tyrosine kinase inhibitors (like PKC412, CEP701 and SU5614) that are known to inhibit FLT3 have become available. When activated FLT3 would occur in JMML, it is conceivable that these patients may benefit from treatment with such inhibitors. Moreover, although effective as a FLT3 inhibitor, PKC412 has originally been developed to inhibit Protein Kinase C (PKC) and thereby interacts with the RAS-RAF-MEK-ERK pathway (19, 20). Therefore, inhibition of PKC by PKC412 may additionally target JMML cells that are characterized by dysregulation of the RAS pathway, which occurs in a large proportion of JMML cells. To investigate whether a subgroup of JMML patients can be identified that might benefit from PKC412 we screened primary JMML samples for the presence of activating *FLT3* mutations, determined *FLT3* and *FLT3 ligand* expression and studied the *in vitro* response to PKC412 in these samples.

PATIENTS AND METHODS

Patients

In this study 51 *de novo* JMML patients were included, after written informed consent of the parents according to the Helsinki agreement. Clinical diagnosis of JMML was established using the diagnostic criteria as described by Niemeyer *et al* (21). Patients were treated according to the guidelines for JMML of the European Working Group of MDS and JMML in Childhood (EWOG-MDS), which basically implies allo SCT. Of these patients 12 (24%) carried a *RAS* mutation, 12 (24%) a *PTPN11* mutation and 1 (2%) a

NF1 mutation. In 17 (33%) these mutations were not found and in 9 (17%) no information about mutations was available. Cryopreserved primary bone marrow (n=28) and/or peripheral blood samples (n=11) were collected at diagnosis. Cells were thawed and resuspended in RMPI 1640 medium (Dutch modification without L-glutamine; 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, 0,125 µg/ml fungizone and 0,2 mg/ml gentamycin (Invitrogen). In addition, from 12 cases leukemic cells were isolated from the spleen as described by Gerhardt *et al* (22) and peripheral blood samples of 23 healthy adults were used as controls. The obtained results were compared with results obtained in leukemic samples from *MLL*-rearranged infant ALL patients and non-infant ALL cases with known FLT3 activation status and PKC412 cytotoxicity data (23).

METHODS

DNA/RNA extraction and quantitive real time-PCR (TaqMan)

Genomic DNA and total RNA were extracted from JMML cells using TRIzol reagent (Invitogen) according to the manufacturer's instructions. Quantification of both RNA and DNA was performed using a spectrophotometer and the integrity of the extracted RNA was assessed on 1% agarose gels.

cDNA was synthesized from 1 µg of total RNA, and the levels of *FLT3* and *FLT3 ligand* expression relative to the expression levels of the endogeneous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured using quantitative real-time PCR (TaqMan) as described previously (24).

Detection of the *FLT3/ITD* mutations

The detection of the *FLT3/ITD* mutations was performed as described by Kiyoi *et al* (6). PCRs were carried out in a total reaction volume of 50 µl containing TaqMan buffer II (Applied Biosystems, Foster City, CA, USA), 300 nM forward and reverse primer, 1.25 U Ampli*Taq* Gold polymerase (Applied Biosystems, Foster City, CA, USA) 2 mM $MgCl_2$, 200 µM of each dNTP (Amersham Pharmacia Biotech, Uppsala, Sweden) and 500 ng of genomic DNA as template. Samples were heated for 10 minutes at 95°C to activate the Ampli*Taq* Gold polymerase and amplified during 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Genomic DNA extracted from the MV4-11 cell line, that has shown to posses a *FLT3/ITD* (25), was used as a positive control.

Detection of *FLT3* activation loop mutations

Detection of the activating mutations affecting Asp835 or Ile836 within the activation loop of the *FLT3* gene was performed essentially as described by Yamamoto *et al* (26). However, as previously described, to fit our standard PCR procedure, a different set of primers was designed to amplify the region of interest (25). To detect mutations, PCR products amplified as described above, were digested overnight at 37°C using the restriction enzyme EcoRV, and seperated on 2% agarose gels to identify possible incomplete digested fragments that suggest the presence of activation loop mutations within the TKD region of *FLT3*.

In vitro PKC412 cytotoxicity (MTT assay)

These analyses were performed as described previously (27). Briefly, JMML cells were cultured in round-bottomed 96-well microtitre plates in the presence of six different concentrations of PKC412 (N-benzoyl staurosporine; kindly provided by Thomas Meyer, Novartis Pharma AG, Basel, Switzerland), with the highest concentration of 10 μM and a 3-fold dilution factor. Control cells were cultured in eight wells in the absence of PKC412. Four wells containing 100 μl culture medium were used as blanks. After incubating the plates for four days at 37°C in humidified air containing 5% CO_2, 10 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT, 5 mg/ml; Sigma) was added and the plates were incubated for an additional six hours under the same conditions. During this final 6 hours incubation, the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals by viable cells only. Formazan crystals were dissolved by adding 100 μl acidified isopropanol (0.04 N HCl-isopropyl alcohol) and the optical density, which is linearly related to the number of viable cells was measured at 562 nm on a spectrophotometer (23).

RESULTS

FLT3 mutation analysis

A total of 51 JMML patients was screened for activating *FLT3* mutations. In none of the JMML samples *FLT3-ITD* mutations nor activating loop point mutations in the *TKD* domain were found.

FLT3 and *FLT3 ligand* expression

In 21 JMML patients the relative expression of *FLT3* and *FLT3 ligand* was determined using quantitative real-time PCR. The results were compared to the expression levels in healthy individuals, non infant ALL patients and *MLL*-rearranged infant ALL patients (Figure 1). The median *FLT3* expression relative to *GAPDH* in JMML patients was 1.46%

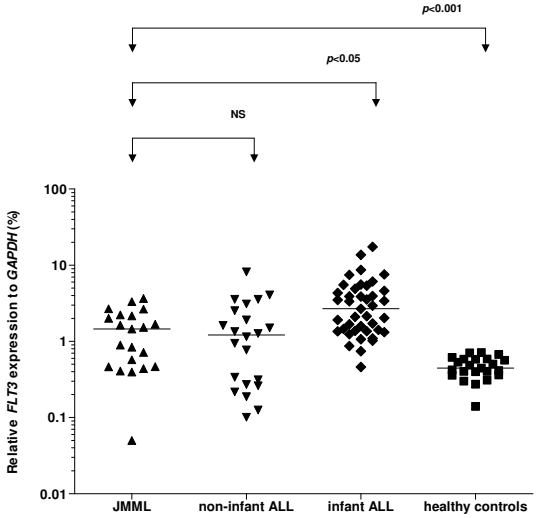


Figure 1: *FLT3* expression in JMML compared to ALL and healthy controls. *FLT3* expression in JMML compared to ALL and healthy controls. The *FLT3* expression in JMML cells (n=21), in non-infant ALL cells (n=22), in infant *MLL*-rearranged ALL cells (n=41) and in cells of healthy control patients (n=23). Statistical test used: Mann Whitney U-test.

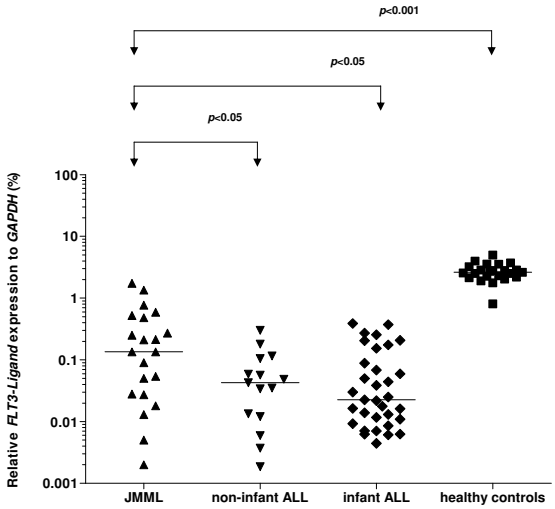


Figure 2: *FLT3 ligand* expression in JMML compared to ALL and healthy controls. *FLT3 ligand* expression in JMML compared with ALL and healthy controls. The *FLT3 ligand* exression in JMML cells (n=21), in non-infant ALL cells (n=15), in infant *MLL*-rearranged ALL cells (n=33) and in cells of healthy control patients (n=23). Statistical test used: Mann Whitney U-test.

(range 0.05-3.65 %) compared to a median *FLT3* expression of 0.45% (range 0.14-0.72%) in healthy controls (p<0.001) and to a median *FLT3* expression in non-infant ALL patients was 1.21% (range 0.10-8.16%, p=0.59). In contrast, in *MLL*-rearranged ALL infants the median level of *FLT3* expression was significantly higher as compared to JMML patients, i.e. 2.67% (range 0.46-17.43%, p<0.05).

In Figure 2 the results of the *FLT3 ligand* expression are depicted. The median relative *FLT3 ligand* expression was 0.14% in the JMML patients (range 0.002-1.74%), 2.62% in the healthy controls (range 0.81-4.99%, p<0.001)), 0.043% in the non infant ALL patients (range 0.002-0.30%, p<0.05) and 0.023% in the *MLL*-rearranged infants (range 0.004-0.388%, p<0.05) respectively (Figure 2).

PKC412 cytotoxicity

In 12 primary JMML samples PKC 412 cytotoxicity was determined. Of these patients 4 carried a *RAS* mutation, 3 a *PTPN11* mutation and 1 a *NF1*-mutation, whereas 4 did not carry these mutations.

No PKC412 cytotoxicity was observed in JMML cells in contrast to cells from *MLL*-rearranged ALL infants with high *FLT3* expression (n=9) in which PKC412 clearly is cytotoxic (Figure 3). Moreover, no significant difference was shown comparing samples from JMML patients with relatively high levels of *FLT3* expression to samples with low levels of *FLT3* expression (Figure 3).

DISCUSSION

JMML is a malignant childhood disease for which, apart from stem cell transplantation, no curative therapy is available so far. After stem cell transplantation a high relapse rate has been reported and eventually 50-60% of the patients will survive (18). This points out the urgent need for drugable targets for the disease in this very young age group.

FLT3 is expressed in a wide variety of both normal and malignant haematopoietic cells (28-34). Mutations constitutively activating FLT3 in a ligand-independent manner

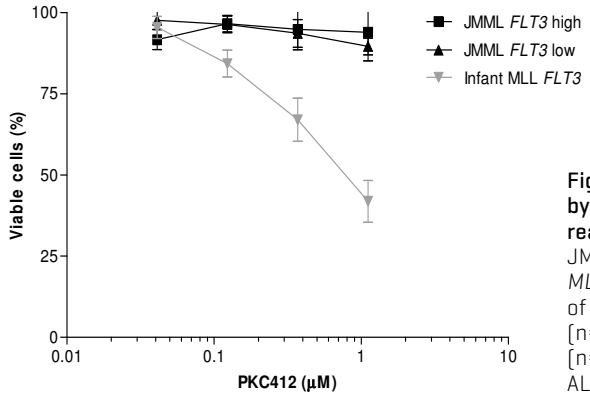


Figure 3: Cytotoxicity of JMML cells by PKC412 as compared to MLL rearranged ALL cells. Cytotoxicity of JMML cells by PKC412 as compared to *MLL*-rearranged ALL cells. Cytotoxicity of JMML cells with high *FLT3* expression (n=6) and with low *FLT3* expression (n=6) compared to *MLL*-rearranged ALL cells (n=9).

have been identified as an important adverse prognostic factor in AML, both in children and adults (6-9, 13, 35). Since in AML activating *FLT3* mutations seem to occur especially in monoblastic subtypes, it is conceivable that activating *FLT3* mutations might play a role in JMML as well. If so, potent small molecule FLT3 inhibitors that are currently being used in Phase I/II studies in adult AML patients may well be effective against JMML.

However, in the present cohort of JMML patients no activating *FLT3* mutations were found. This is consistent with a recent study by Gratias *et al*, who found only one patient with a C836G mutation in a cohort of 60 JMML patients (36), as well as with a study by Xu *et al* who found no mutations among 15 children with juvenile chronic myelogenous leukemia (12).

Apart from activating by mutations, FLT3 can also become constitutively activated in a ligand-independent manner merely by overexpression of the wild-type receptor as was shown in *MLL*-rearranged infant ALL patients (23, 37). In addition, Zheng *et al* reported evidence of autocrine signaling in AML cells co-expressing FLT3 and *FLT3 ligand* (17). In contrast, in our JMML patients neither *FLT3* nor *FLT3 ligand* overexpression was found. This was consistent with the fact that cytoxicity was not observed at clinically relevant concentrations of PKC412 like in *MLL*-rearranged infant ALL patients, even not in JMML patients with the highest *FLT3* expression levels (Figure 3) (38). These data strongly suggest that FLT3 is not constitutively activated and that apparently, FLT3 activation does not play a role in JMML. One could wonder what determines the difference between the role of FLT3 in leukemogenesis in JMML and AML, where mutations predominantly are found in M4/5. The FLT3 receptor is expressed by normal haematopoietic progenitors but seems to be restricted to the earliest stages (34). In JMML the origin has been traced in the early myeloid progenitor and the pluripotent stem cell, but these cells are capable of terminal differentiation. The fact that primary JMML samples are composed of only small percentages of abnormal early progenitors and that *FLT3* expression is lost during differentiation may explain the relatively low *FLT3* expression in JMML as compared to acute leukemia (36).

As PKC412 is developed as a protein kinase inhibitor, PKC412 also interferes with the RAS-RAF-MEK-ERK pathway, and not only by inhibiting FLT3 directly. Therefore inhibition of PKC by PKC412 may additionally target JMML cells that are characterized by dysregulation of the RAS pathway, which occurs in the vast majority of the JMML cells

[19]. We showed that the inhibition of this pathway did not result in cytotoxicity, not even in the *RAS*-pathway mutated cases, indicating that blocking RAS by protein kinase inhibition is not useful in JMML.

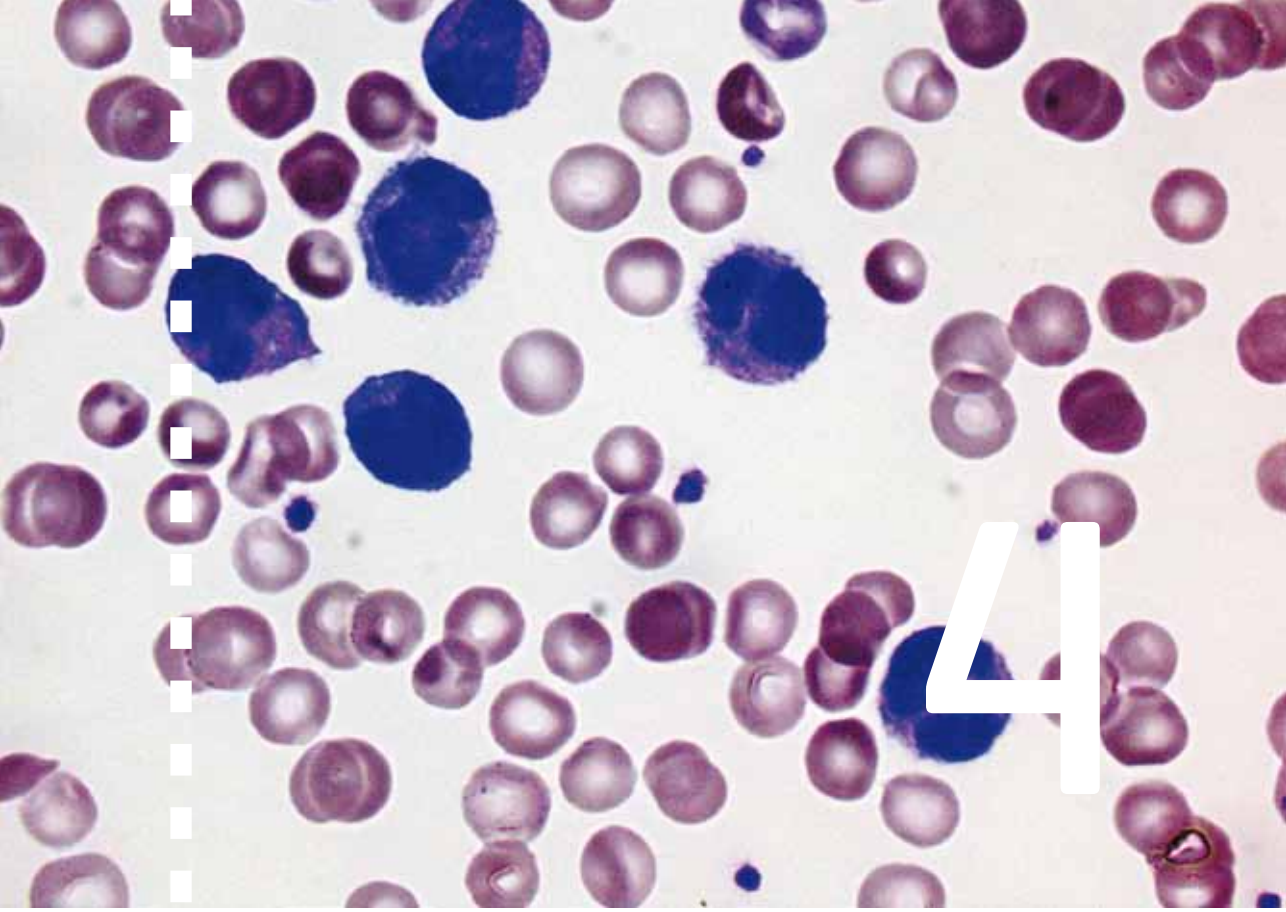
In summary, our study shows that constitutively activated FLT3 does not occur in JMML, nor by mutations, by overexpression of the wild-type FLT3 or by autocrine signaling by FLT3 ligand. This implicates that it is very unlikely that patients with JMML will benefit from treatment with FLT3 inhibitors.

REFERENCES

1. Kalra R, Paderanga DC, Olson K, Shannon KM. Genetic analysis is consistent with the hypothesis that NF1 limits myeloid cell growth through p21ras. Blood. 1994 Nov 15;84(10):3435-9.
2. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J, Jung A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat Genet. 2003 Jun;34(2):148-50.
3. Side LE, Emanuel PD, Taylor B, Franklin J, Thompson P, Castleberry RP, et al. Mutations of the NF1 gene in children with juvenile myelomonocytic leukemia without clinical evidence of neurofibromatosis, type 1. Blood. 1998 Jul 1;92(1):267-72.
4. Beaupre DM, Kurzrock R. RAS and leukemia: from basic mechanisms to gene-directed therapy. J Clin Oncol. 1999 Mar;17(3):1071-9.
5. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996 Dec;10(12):1911-8.
6. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999 May 1;93(9):3074-80.
7. Kondo M, Horibe K, Takahashi Y, Matsumoto K, Fukuda M, Inaba J, et al. Prognostic value of internal tandem duplication of the FLT3 gene in childhood acute myelogenous leukemia. Med Pediatr Oncol. 1999 Dec;33(6):525-9.
8. Meshinchi S, Woods WG, Stirewalt DL, Sweetser DA, Buckley JD, Tjoa TK, et al. Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. Blood. 2001 Jan 1;97(1):89-94.
9. Rombouts WJ, Blokland I, Lowenberg B, Ploemacher RE. Biological characteristics and prognosis of adult acute myeloid leukemia with internal tandem duplications in the Flt3 gene. Leukemia. 2000 Apr;14(4):675-83.
10. Stirewalt DL, Kopecky KJ, Meshinchi S, Appelbaum FR, Slovak ML, Willman CL, et al. FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. Blood. 2001 Jun 1;97(11):3589-95.
11. Stirewalt DL, Meshinchi S, Kussick SJ, Sheets KM, Pogosova-Agadjanyan E, Willman CL, et al. Novel FLT3 point mutations within exon 14 found in patients with acute myeloid leukaemia. Br J Haematol. 2004 Feb;124(4):481-4.
12. Xu F, Taki T, Yang HW, Hanada R, Hongo T, Ohnishi H, et al. Tandem duplication of the FLT3 gene is found in acute lymphoblastic leukaemia as well as acute myeloid leukaemia but not in myelodysplastic syndrome or juvenile chronic myelogenous leukaemia in children. Br J Haematol. 1999 Apr;105(1):155-62.
13. Zwaan CM, Meshinchi S, Radich JP, Veerman AJ, Huismans DR, Munske L, et al. FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. Blood. 2003 Oct 1;102(7):2387-94.
14. Nicola NA. Hemopoietic cell growth factors and their receptors. Annu Rev Biochem. 1989;58:45-77.
15. Choudhary C, Muller-Tidow C, Berdel WE, Serve H. Signal transduction of oncogenic Flt3. Int J Hematol. 2005 Aug;82(2):93-9.
16. Ishiko J, Mizuki M, Matsumura I, Shibayama H, Sugahara H, Scholz G, et al. Roles of tyrosine residues 845, 892 and 922 in constitutive activation of murine FLT3 kinase domain mutant. Oncogene. 2005 Aug 1.
17. Zheng R, Levis M, Piloto O, Brown P, Baldwin BR, Gorin NC, et al. FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. Blood. 2004 Jan 1;103(1):267-74.
18. Locatelli F, Niemeyer C, Angelucci E, Bender-Gotze C, Burdach S, Ebell W, et al. Allogeneic bone marrow transplantation for chronic myelomonocytic leukemia in childhood: a report from the European Working Group on Myelodysplastic Syndrome in Childhood. J Clin Oncol. 1997 Feb;15(2):566-73.
19. Kong AN, Yu R, Chen C, Mandlekar S, Primiano T. Signal transduction events elicited by natural products: role of MAPK and caspase pathways in homeostatic response and induction of apoptosis. Arch Pharm Res. 2000 Feb;23(1):1-16.
20. Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer. 2003 Sep;3(9):650-65.
21. Niemeyer CM, Arico M, Basso G, Biondi A, Cantu Rajnoldi A, Creutzig U, et al. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. European Working Group on

Myelodysplastic Syndromes in Childhood (EWOG-MDS). Blood. 1997 May 15;89(10):3534-43.

22. Gerhardt TM, Schmahl GE, Flotho C, Rath AV, Niemeyer CM. Expression of the Evi-1 gene in haemopoietic cells of children with juvenile myelomonocytic leukaemia and normal donors. Br J Haematol. 1997 Dec;99(4):882-7.
23. Stam RW, den Boer ML, Schneider P, Nollau P, Horstmann M, Beverloo HB, et al. Targeting FLT3 in primary MLL-gene-rearranged infant acute lymphoblastic leukemia. Blood. 2005 Oct 1;106(7):2484-90.
24. Stam RW, den Boer ML, Meijerink JP, Ebus ME, Peters GJ, Noordhuis P, et al. Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood. 2003 Feb 15;101(4):1270-6.
25. Levis M, Allebach J, Tse KF, Zheng R, Baldwin BR, Smith BD, et al. A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo. Blood. 2002 Jun 1;99(11):3885-91.
26. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001 Apr 15;97(8):2434-9.
27. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003 Sep 1;21(17):3262-8.
28. Birg F, Courcoul M, Rosnet O, Bardin F, Pebusque MJ, Marchetto S, et al. Expression of the FMS/KIT-like gene FLT3 in human acute leukemias of the myeloid and lymphoid lineages. Blood. 1992 Nov 15;80(10):2584-93.
29. Brasel K, Escobar S, Anderberg R, de Vries P, Gruss HJ, Lyman SD. Expression of the flt3 receptor and its ligand on hematopoietic cells. Leukemia. 1995 Jul;9(7):1212-8.
30. DaSilva N, Hu ZB, Ma W, Rosnet O, Birnbaum D, Drexler HG. Expression of the FLT3 gene in human leukemia-lymphoma cell lines. Leukemia. 1994 May;8(5):885-8.
31. Drexler HG. Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. Leukemia. 1996 Apr;10(4):588-99.
32. Kainz B, Heintel D, Marculescu R, Schwarzinger I, Sperr W, Le T, et al. Variable prognostic value of FLT3 internal tandem duplications in patients with de novo AML and a normal karyotype, t(15;17), t(8;21) or inv(16). Hematol J. 2002;3(6):283-9.
33. Meierhoff G, Dehmel U, Gruss HJ, Rosnet O, Birnbaum D, Quentmeier H, et al. Expression of FLT3 receptor and FLT3-ligand in human leukemia-lymphoma cell lines. Leukemia. 1995 Aug;9(8):1368-72.
34. Rosnet O, Schiff C, Pebusque MJ, Marchetto S, Tonnelle C, Toiron Y, et al. Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. Blood. 1993 Aug 15;82(4):1110-9.
35. Goemans BF, Zwaan CM, Miller M, Zimmermann M, Harlow A, Meshinchi S, et al. Mutations in KIT and RAS are frequent events in pediatric core-binding factor acute myeloid leukemia. Leukemia. 2005 Sep;19(9):1536-42.
36. Gratias EJ, Liu YL, Meleth S, Castleberry RP, Emanuel PD. Activating FLT3 mutations are rare in children with juvenile myelomonocytic leukemia. Pediatr Blood Cancer. 2005 Feb;44(2):142-6.
37. Armstrong SA, Kung AL, Mabon ME, Silverman LB, Stam RW, Den Boer ML, et al. Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. Cancer Cell. 2003 Feb;3(2):173-83.
38. Brown P, Levis M, Shurtleff S, Campana D, Downing J, Small D. FLT3 inhibition selectively kills childhood acute lymphoblastic leukemia cells with high levels of FLT3 expression. Blood. 2005 Jan 15;105(2):812-20.



4

RELEVANCE OF *WT1* EXPRESSION, MUTATIONS AND SINGLE NUCLEOTIDE POLYMORPHISMS IN JUVENILE MYELOMONOCYTIC LEUKEMIA

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Submitted

Illustration: JMML, monocytosis and abnormal monocytes (PB)

ABSTRACT

Approximately 85% of patients with juvenile myelomonocytic leukemia (JMML) harbour mutations in genes involved in the RAS-RAF-MEK-ERK pathway. In the remaining 15% of the cases unknown alternative molecular aberrations may be involved. In patients with acute leukemia and myelodysplastic syndrome increased Wilms' tumor gene (*WT1)* expression has been reported, in part as a result of *WT1* mutations.

Similarly, in JMML, *WT1* expression has been reported to be significantly higher than in normal bone marrow. To date, it is unknown whether *WT1* upregulation is caused by mutations in the *WT1* gene and whether such mutations play a role in the JMML leukemogenesis.

We performed quantitative *WT1* expression analysis in 58 JMML patients by RQ-PCR and screened the whole WT1 gene for mutations by direct sequencing in 48 cases.

Median *WT1* expression levels in JMML were high compared to healthy controls, but not as high as in pediatric AML patients. *WT1* overexpression in JMML was not correlated to mutation status or SNPs. In 2/48 JMML patients *WT1* mutations were found: one patient harboured a mutation in exon 1 and one patient harboured two mutations in exon 7. SNPS were found in exon 1 (rs2234581: 15/48; rs5030136: 4/48), exon 3 (rs1799933: 9/48), exon 6 (rs2234590: 2/48) and exon 7 (rs16754: 14/48). *WT1* expression levels of the samples with mutations or with SNPs did not differ from the wild type cases. No influence of *WT1* expression nor SNPs was found on overall survival.

In conclusion, *WT1* upregulation in JMML is lower than in pediatric AML, is not regulated by mutations or SNPs, and does not influence outcome in JMML.

INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) is a rare malignant disease in young children, which accounts for less than 3% of all childhood hematological malignancies. The disease is characterized by prominent hepatosplenomegaly, absolute monocytosis, presence of myeloid precursors in peripheral blood, low platelet count, frequent skin involvement and *in vitro* granulocyte-macrophage colony stimulating factor (GM-CSF) hypersensitivity (1). The proliferation advantage of JMML cells is caused by continuous activation of the GM-CSF-receptor-RAS-RAF-MEK-ERK signal transduction pathway caused by activating mutations of *RAS* (25% of the cases) (2) or *PTPN11* (35% of the cases) (3-5). In addition, 11% of the JMML cases are associated with clinical Neurofibromatosis type 1 (NF1) or bi-allelic inactivation of the *NF1* tumor suppressor gene (6-7). Recently, germline mutations in *c-CBL* were identified in JMML patients, which also result in a continuous activation of RAS (8-9). Still, in about 15% of the JMML cases no RAS pathway activating mutations are found, suggesting that other biological mechanisms play a role (10-12).

A candidate gene that might play a role in the pathogenesis of JMML is the Wilms' tumor 1 gene (*WT1*). In JMML patients, *WT1* expression has been found to be significantly high as compared to normal bone marrow (13). Similarly, in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) increased levels of *WT1* expression have been reported in a large proportion of the patients, especially in normal karyotype AML, which include a high frequency of M4/M5 leukemias (monocytic and myelomonocytic leukemias) (13-14). Recent studies showed that in childhood AML *WT1* is also upregulated in cases with mutations in the *WT1* gene (14-15). In addition, Damm *et al* identified the SNP rs16754 in the *WT1* gene as an independent favourable risk factor in normal karyotype adult AML. Data about the prognostic value of rs16754 in childhood AML are conflicting (16-18).

Interestingly, the potential of *WT1* to serve as a target for immunotherapy showed promising results in leukemia and solid tumors which may be relevant for JMML, a disease for which so far, apart from hematopoietic stem cell transplantation (HSCT) no therapy is available (19-20).

However, to date in JMML it is not known which *WT1* aberrations regulate overexpression nor whether disease progression or outcome in JMML is associated with upregulation. For that reason, we conducted whole *WT1* gene sequencing in correlation with *WT1* expression and outcome analysis in JMML patients.

PATIENTS AND METHODS

Patients

In this study, 36 JMML patients were included for mutation and expression analysis, and an additional 12 patients were included for mutation analysis only. For the survival analysis we included 22 patients of which the expression data are previously described (13). Informed consent from the parents was obtained according to the Helsinki declaration (59th WMA General Assembly, Seoul, October 2008). The diagnosis of JMML was based on previously described criteria, and all patients were registered and treated according to the guidelines for JMML of the European Working Group of MDS and JMML in Childhood (EWOG- MDS) (21-23).

Of the total cohort of 70 patients nine patients had a clinical diagnosis of NF1, 18 carried a somatic mutation in *NRAS* or *KRAS*, 17 patients had a somatic mutation of *PTPN11* and 3 patients harboured a *c-CBL* mutation. In 11 cases no known mutation was found and in 12 cases no information was available.

Samples

DNA and RNA were isolated from mononuclear cells derived from cryopreserved primary bone marrow, peripheral blood or spleen at diagnosis as previously described (24). Briefly, cells were thawed and resuspended in RMPI 1640 medium (Dutch modification without L-glutamine; 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, 0,125 µg/ml fungizone and 0,2 mg/ml gentamycin (Invitrogen). Genomic DNA was extracted from a minimum of 5 x 10^6 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

WT1 expression analysis

For quantitative expression analysis, quantitative RT-PCR was performed as previously described (14, 25). The *ABL* gene was chosen as a control (housekeeping) gene to evaluate the amount and amplification of cDNA as its transcript represents a reliable control gene according to the recommendations of the EAC program (26). *WT1* overexpression was defined as expression levels higher than the lowest *WT1* expression in childhood AML patients.

Whole *WT1* gene sequencing

To investigate the presence of mutations in exon 1 through 6, the purified DNA was subjected to 41 cycles of PCR using a touchdown approach of 30 minutes at 94 °C, 30 minutes at 66-54 °C (1x 66 °C, 2 x 64 °C, 3x 62 °C, 4x 60 °C, 5x 58 °C, 6x 56 °C, and 20 x at 54 °C) and 30 minutes at 72 °C using (M13-tagged) using previous described primers. PCR products were directly sequenced unidirectionally using M13-primers and analyzed using Codoncode aligner (Codoncode, Dedham, MA, USA). Mutations were confirmed by an independent amplification of the fragment and direct sequencing of both strands as previously described (27).

For mutation analysis of exons 7 through 10 of the *WT1* gene genomic DNA was PCR amplified using specific primers as previous described (27). The following PCR conditions were used: 2 minutes at 50 °C, 10 minutes at 95 °C, 40 cycles f 15 minutes at 95 °C and 1 minute at 60 °C, and an extension step of 10 minutes at 72 °C as previously described (27). Purified PCR products were directly sequenced from both strands using the described primers. The sequence data were analyzed using CLC Workbench version 3.5.1 (CLCBio, Aarhus, Denmark).

Mutation analysis of other RAS-pathway activating aberrations

To determine the mutational status of the JMML samples, analysis of RAS pathway activating aberrations, including *PTPN11, NRAS, KRAS, BRAF* and *c-CBL* was performed as previously described (3, 8, 10, 28). Information on clinical signs of Neurofibromatosis type 1 was collected from the EWOG-MDS database.

RESULTS

WT1 expression levels

The median *WT1* expression in JMML patients was 106/1x 10^4 *ABL* copies (range 0-2700/1x 10^4 *ABL* copies) compared with a median expression of 18/1x 10^4 *ABL* copies (range 0-400/1x 10^4 *ABL* copies) in healthy controls (p=0.006). The median level of *WT1* expression in JMML patients was significantly lower than in pediatric AML patients, i.e. 4700/1x 10^4 *ABL* copies (range 400-36000/1x 10^4 *ABL* copies, p < 0.0001) (Figure 1). In 9/58 (15%) *WT1* expression was as high as in AML patients. *WT1* expression levels in the JMML patients were significantly lower compared to the AML M4/M5 cases.

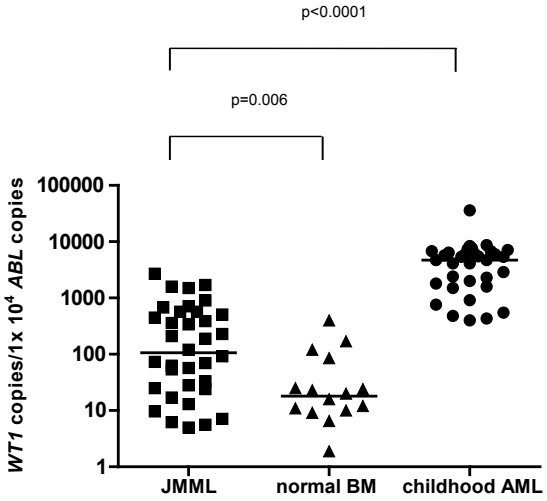


Figure 1: WT1 expression in JMML patients as compared to WT1 expression levels in childhood AML and normal bone marrow. *WT1* expression in JMML (n=36) compared with healthy controls (n=16) and childhood AML patients (n=34). Statistical test used: Mann Whitney U-test. The horizontal line represents the median expression levels of the different groups.

No significant difference in *WT1* expression was found between the different RAS-pathway mutational subgroups of JMML (Median *WT1* expression levels in JMML patients with a *RAS* mutation: 360/1x 10^4 *ABL* copies (range 0-570/1x 10^4 *ABL* copies), *PTPN11* mutation: 230/1x 10^4 *ABL* copies (range 24-1600/1x 10^4 *ABL* copies), clinical NF1 cases: 91/1x 10^4 *ABL* copies (range 9.7-510/1x 10^4 *ABL* copies)). No *WT1* expression levels were available of the patients with c-CBL mutations. The median *WT1* expression of the patients without a RAS pathway mutation was 54/1x 10^4 *ABL* copies (range 0-570/1x 10^4 *ABL* copies). Furthermore, no correlation was found between the *WT1* expression levels and white blood cell count (Spearman r= -0.05) or age at diagnosis (Spearman r= 0.1).

WT1 whole gene sequencing

WT1 whole gene sequencing was performed in 48 patients. Two patients harboured a mutation in the *WT1* gene. One patient showed a heterozygeous mutation in exon 1: c191delC., resulting in amino acid substitution. This patient did not carry a mutation in *NF1, c-CBL, RAS* or *PTPN11*. The second patient showed two heterozygeous mutations in exon 7 of the *WT1* gene: 10C>T (R370C) and 136A>G (S412G) (Table 1, Figure 2). This patient carried also a *PTPN11* mutation.

Single nucleotide polymorphisms (SNPs) were found in exon 1, 3, 6 and 7 (Table 1). Rs2234581 (exon 1) was found in 15/48 cases, (homozygeous in three cases). Rs5030136 (exon 1) was found in 4/48 cases (all heterozygeous), rs1799933 (exon 3) in 9/48 cases (all heterozygeous) and rs2234590 (exon6) in 2/48 cases (all heterozygeous). Fourteen (29.1%) of the 48 patients carried the *WT1* SNP rs16754 (exon 7), all heterozygeous. No differences in *WT1* expression aberrations was found between patients with or without SNPs, nor between patients carrying any of the individual SNPs as compared to the other SNPs or mutations in the *WT1* gene (Table 1).

WT1 and outcome

In this cohort 9/58 (15%) patients had *WT1* expression in the range of pediatric AML patients (='high'). No significant difference was found in overall survival between the patients with high WT1 expression compared to the patients with normal expression levels (log rank p=0.2) (Figure 3). Patients who relapsed after transplantation (7/58, 12%) showed no significant higher *WT1* expression at diagnosis compared to patient without relapse.

We identified *WT1* gene mutations in only 2/48 JMML patients (4.1%). One patients deceased 1.1 year after stem cell transplantation due to therapy related toxicity. The other patient is alive thirteen year after stem cell transplantation.

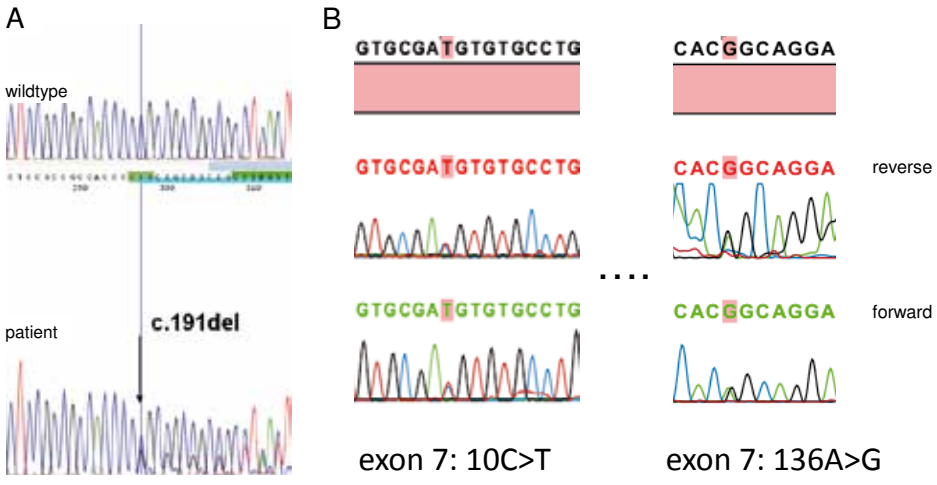


Figure 2: Mutations found in the WT1 gene. *WT1* gene mutations found JMML patients. Panel A: Patient A harbouring harboring a mutation in exon 1: c.191delC. Panel B: Patient B harbouring two mutations in exon 7, at the left side 10C>T and at the right side 136 A>G

Table 1: Clinical characteristics of the JMML patients harbouring a mutation or SNP in the WT1 gene.

	c191delC	10 C>T (R370C) 136 A>G(S412G)	rs2234581	rs5030136	rs1799933	rs2234590	rs16754
Exon	7	7	1	1	3	6	7
Frequency	1/48	1/48	15/48	4/48	9/48	2/48	14/48
Gender (male/female)	1/0	1/0	9/6	4/0	7/2	2/0	10/4
Median age at diagnosis (years) (range)	0.41	4.58	1.75 (0.25-5.91)	2.0 (0.5-5.91)	1.5 (0.25-5.91)	0.875 (0.25-1.5)	1.62 (0.083-4.66)
WBC (x 10^9/l) median (range) Blast% PB	16.2 0	39.6 0	31 (4.0-88.2) 2 (0-12)	41 (11-56.7) 6 (0-12)	36.5 (11-56.7) 2 (0-12)	33.5 (32-35) 2 (1-3)	32 (11-74.6) 5 (0-14)
RAS-pathway mutation							
- no	1		3	1	2	1	2
- RAS			3	1	3	1	0
- PTPN11		1	5	1	1	0	5
- NF1			0	0	0	0	3
- c-CBL			0	0	0	0	0
- na			4	1	3	0	4
Median WT1-expression (/10^4 ABL copies) (range)			82.5 (0-1700)	796 (28-1700)	92 (3-1700)	355 (0-710)	77 (0-1491)
Outcome (alive/death)	1/0	0/1	8/7	2/2	4/5	1/1`	7/7
5-years overall survival (%)			50	50	44	50	49

WBC = white blood cell count
PB = peripheral blood
na = not available

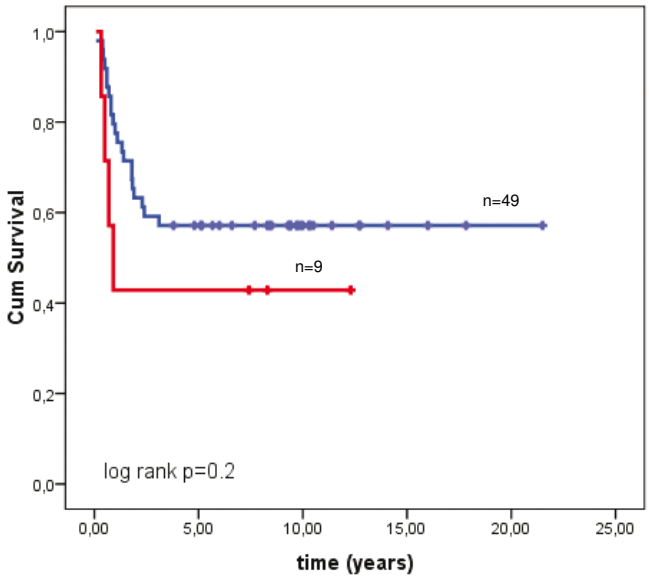


Figure 3: Overall survival of JMML patients with high and normal *WT1* expression. Kaplan Meier plot representing the overall survival of JMML patients with high *WT1* expression [—] compared to the JMML patients with normal *WT1* expression [—]

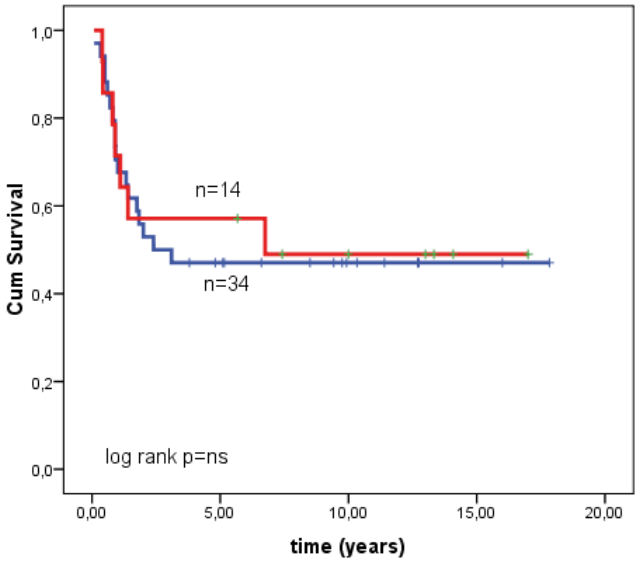


Figure 4: Overall survival of JMML patients with the rs16754 SNP in the *WT1* gene versus patients with wildtype *WT1* gene. Kaplan Meier plot representing the overall survival of JMML carrying rs16754 [—] compared to the JMML patients without rs16754 [—]

As rs16754 in AML might be seen as a prognostic factor we looked at the influence of the occurrence of this SNP on outcome. Figure 4 shows the overall survival curve of the fourteen JMML patients carrying rs16754 (SNP) compared to the wildtype patients. We did not find a significantly difference in overall survival between these two groups (overall survival 49% vs. 47%, log rank: NS).

No difference in overall survival was found for the patients carrying rs2234581, rs5030136, rs1799933 or rs2234590 compared to wildtype patients (Table 1).

DISCUSSION

This study shows that *WT1* is overexpressed in JMML patients and the upregulation is not caused by mutations or SNPs in the *WT1* gene. Nevertheless, *WT1* overexpression in JMML interestingly does not reach prognostic relevant expression levels we found in pediatric AML patients (13-14).

WT1 overexpression may therefore encourage development of new therapeutic strategies, i.e. targeting *WT1* by immunotherapy as described in adult AML in a subset of the JMML patients (19-20, 29).

We showed no difference in overall survival between JMML cases with a high *WT1* expression levels versus those with lower *WT1* expression. In AML conflicting data are published about the relevance of *WT1* expression levels on outcome (14, 17, 30-37). This suggests that *WT1* has a less dominant influence on leukemogenesis in JMML than in AML.

Until now no data were available about the regulation of the *WT1* overexpression in JMML. The present study shows that in JMML *WT1* expression is not regulated by mutations or SNPs in the *WT1* gene, as in only 2/48, low *WT1* expressing, JMML patients a *WT1* mutation was found (4.2%). In pediatric normal karyotype AML *WT1* mutations are found in 22% of the cases (17, 27). These mutated AML cases express significantly higher *WT1* levels than wild type cases (14-15, 17, 38). In the JMML patients SNPs were found in exon 1, 3, 6 and 7 of the *WT1* gene, in a frequency which is equal to data described in the ensemble database (www.ensembl.org) and without subsequent *WT1* overexpression. This in contrast to adult AML studies in which rs16754 (exon 7) resulted in overexpression of *WT1*. Pediatric AML studies showed conflicting results on the influence of SNPs in the *WT1* gene on the mRNA expression levels (15-17).

Recently, adult studies reported a favorable outcome for normal karyotype AML with a specific single nucleotide polymorphism (rs16754) of the *WT1* gene. In pediatric AML cohorts data are conflicting. We showed no survival benefit of patients carrying rs16754, which is in contrast to the data found by Ho *et al* who found an increased overall survival of the patients with a favorable karyotype with rs16754 (17-18). The present study demonstrated that there is no relation between the presence of rs16754 or other SNPs in the *WT1* gene and survival in JMML.

It remains to be determined why *WT1* plays a minor role in JMML compared to pediatric AML. It is conceivable that hyperproliferation, rather than maturation arrest, is the hallmark in JMML, may in part explain this difference. Biological studies showed that *WT1* is downregulated as an early event in differentiation during normal haematopoiesis (39-40). Normal bone marrow progenitor cells are refractory to differentiation when they were retrovirally transduced with *WT1* (41-43). It was suggested that overexpression of *WT1* at an early stage of haematopoiesis blocked differentiation and supports the

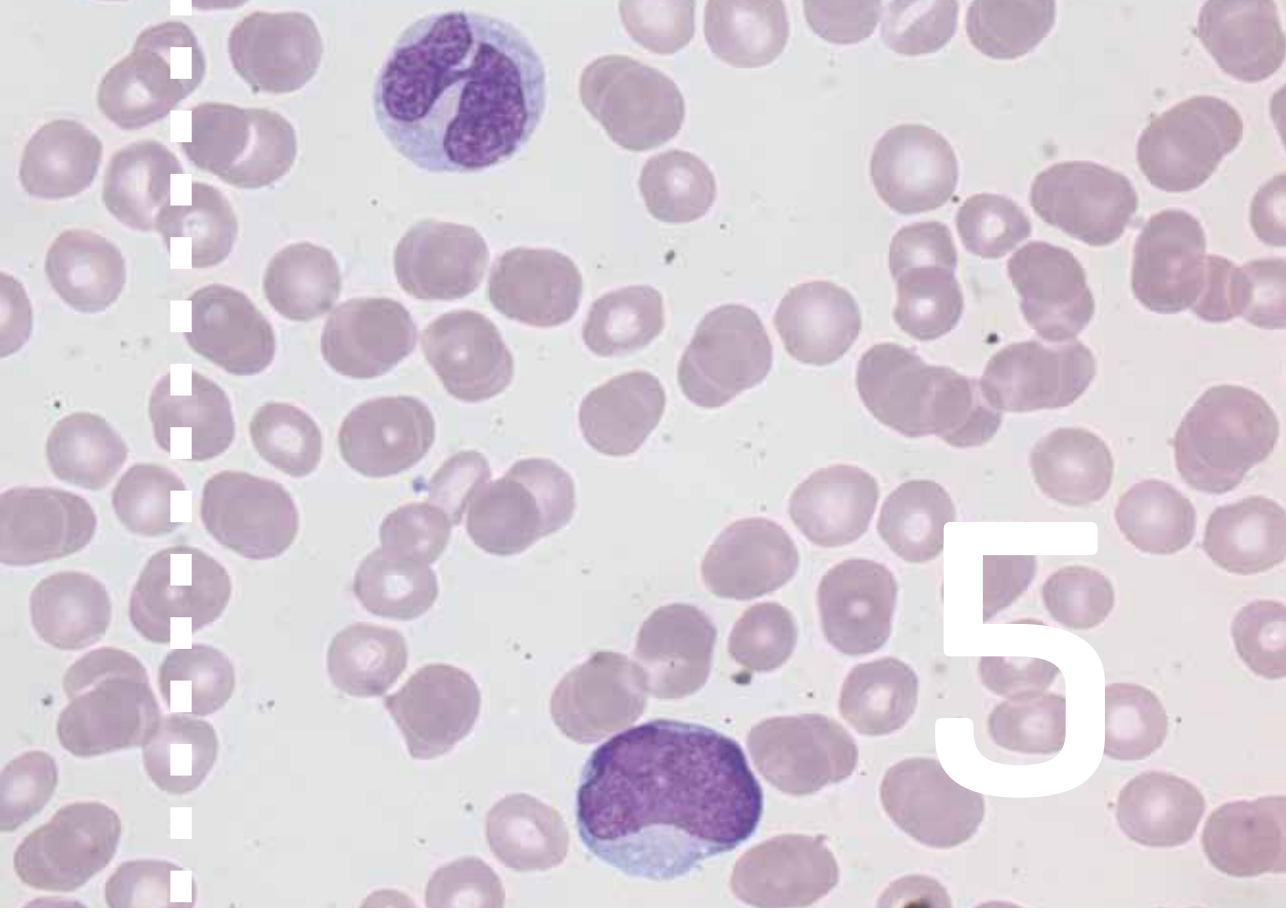
maintenance of progenitor cells in a state of continuing proliferation rather than stimulating them and regulated by interaction with transcription factors (41-43).

In conclusion, in JMML, *WT1* mutations are rare and *WT1* overexpression is not regulated by mutations nor by SNPs in the *WT1* gene. Furthermore, *WT1* overexpression does not have prognostic impact. Further studies are necessary to determine the role of *WT1* overexpression in subsets of JMML patients, and to investigate whether subgroups of JMML patients may benefit from *WT1* inhibition treatment.

REFERENCES

1. Emanuel PD, Bates LJ, Castleberry RP, Gualtieri RJ, Zuckerman KS. Selective hypersensitivity to granulocyte-macrophage colony-stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors. Blood. 1991 Mar 1;77(5):925-9.
2. Kalra R, Paderanga DC, Olson K, Shannon KM. Genetic analysis is consistent with the hypothesis that NF1 limits myeloid cell growth through p21ras. Blood. 1994 Nov 15;84(10):3435-9.
3. Kratz CP, Niemeyer CM, Castleberry RP, Cetin M, Bergstrasser E, Emanuel PD, et al. The mutational spectrum of PTPN11 in juvenile myelomonocytic leukemia and Noonan syndrome/myeloproliferative disease. Blood. 2005 Sep 15;106(6):2183-5.
4. Loh ML, Vattikuti S, Schubbert S, Reynolds MG, Carlson E, Lieuw KH, et al. Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis. Blood. 2004 Mar 15;103(6): 2325-31.
5. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J, Jung A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat Genet. 2003 Jun;34(2):148-50.
6. Bollag G, Clapp DW, Shih S, Adler F, Zhang YY, Thompson P, et al. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. Nat Genet. 1996 Feb;12(2):144-8.
7. Side LE, Emanuel PD, Taylor B, Franklin J, Thompson P, Castleberry RP, et al. Mutations of the NF1 gene in children with juvenile myelomonocytic leukemia without clinical evidence of neurofibromatosis, type 1. Blood. 1998 Jul 1;92(1):267-72.
8. Loh ML, Sakai DS, Flotho C, Kang M, Fliegauf M, Archambeault S, et al. Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. Blood. 2009 Aug 27;114(9):1859-63.
9. Schmidt MH, Dikic I. The Cbl interactome and its functions. Nature reviews. 2005 Dec;6(12):907-18.
10. de Vries AC, Stam RW, Kratz CP, Zenker M, Niemeyer CM, van den Heuvel-Eibrink MM. Mutation analysis of the BRAF oncogene in juvenile myelomonocytic leukemia. Haematologica. 2007 Nov;92(11):1574-5.
11. de Vries AC, Stam RW, Schneider P, Niemeyer CM, van Wering ER, Haas OA, et al. Role of mutation independent constitutive activation of FLT3 in juvenile myelomonocytic leukemia. Haematologica. 2007 Nov;92(11):1557-60.
12. Kratz CP, Niemeyer CM, Thomas C, Bauhuber S, Matejas V, Bergstrasser E, et al. Mutation analysis of Son of Sevenless in juvenile myelomonocytic leukemia. Leukemia. 2007 May;21(5):1108-9.
13. Bader P, Niemeyer C, Weber G, Coliva T, Rossi V, Kreyenberg H, et al. WT1 gene expression: useful marker for minimal residual disease in childhood myelodysplastic syndromes and juvenile myelomonocytic leukemia? European journal of haematology. 2004 Jul;73(1):25-8.
14. Willasch AM, Gruhn B, Coliva T, Kalinova M, Schneider G, Kreyenberg H, et al. Standardization of WT1 mRNA quantitation for minimal residual disease monitoring in childhood AML and implications of WT1 gene mutations: a European multicenter study. Leukemia. 2009 Aug;23(8):1472-9.
15. Ho PA, Kuhn J, Gerbing RB, Pollard JA, Zeng R, Miller KL, et al. WT1 synonymous single nucleotide polymorphism rs16754 correlates with higher mRNA expression and predicts significantly improved outcome in favorable-risk pediatric acute myeloid leukemia: a report from the children's oncology group. J Clin Oncol. 2011 Feb 20;29(6):704-11.
16. Damm F, Heuser M, Morgan M, Yun H, Grosshennig A, Gohring G, et al. Single nucleotide polymorphism in the mutational hotspot of WT1 predicts a favorable outcome in patients with cytogenetically normal acute myeloid leukemia. J Clin Oncol. 2010 Feb 1;28(4):578-85.
17. Hollink IH, van den Heuvel-Eibrink MM, Zimmermann M, Balgobind BV, Arentsen-Peters ST, Alders M, et al. No prognostic impact of the WT1 gene single nucleotide polymorphism rs16754 in pediatric acute myeloid leukemia. J Clin Oncol. 2010 Oct 1;28(28):e523-6; author reply e7-e8.
18. Ho PA, Kuhn J, Gerbing RB, Pollard JA, Zeng R, Miller KL, et al. The WT1 synonymous SNP rs 16754 correlates with higher mRNA expression and predicts significantly improved outcome in favorable-risk pedatric AML: a report of the Children's ncology Group. in press.
19. Weber G, Karbach J, Kuci S, Kreyenberg H, Willasch A, Koscielniak E, et al. WT1 peptide-specific T cells generated from peripheral blood of healthy donors: possible implications for adoptive immunotherapy after allogeneic stem cell transplantation. Leukemia. 2009 Sep;23(9):1634-42.
20. Gao L, Bellantuono I, Elsasser A, Marley SB, Gordon MY, Goldman JM, et al. Selective elimination of leukemic

CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. Blood. 2000 Apr 1;95(7):2198-203.
21. Chan RJ, Cooper T, Kratz CP, Weiss B, Loh ML. Juvenile myelomonocytic leukemia: a report from the 2nd International JMML Symposium. Leukemia research. 2009 Mar;33(3):355-62.
22. de Vries AC, Zwaan CM, van den Heuvel-Eibrink MM. Molecular basis of juvenile myelomonocytic leukemia. Haematologica. Feb;95(2):179-82.
23. Niemeyer CM, Arico M, Basso G, Biondi A, Cantu Rajnoldi A, Creutzig U, et al. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). Blood. 1997 May 15;89(10):3534-43.
24. Gerritsen A, Lam K, Marion Schneider E, van den Heuvel-Eibrink MM. An exclusive case of juvenile myelomonocytic leukemia in association with Kikuchi's disease and hemophagocytic lymphohistiocytosis and a review of the literature. Leukemia research. 2006 Oct;30(10):1299-303.
25. Kreuzer KA, Saborowski A, Lupberger J, Appelt C, Na IK, le Coutre P, et al. Fluorescent 5'-exonuclease assay for the absolute quantification of Wilms' tumour gene (WT1) mRNA: implications for monitoring human leukaemias. Br J Haematol. 2001 Aug;114(2):313-8.
26. Beillard E, Pallisgaard N, van der Velden VH, Bi W, Dee R, van der Schoot E, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. Leukemia. 2003 Dec;17(12):2474-86.
27. Hollink IH, van den Heuvel-Eibrink MM, Zimmermann M, Balgobind BV, Arentsen-Peters ST, Alders M, et al. Clinical relevance of Wilms tumor 1 gene mutations in childhood acute myeloid leukemia. Blood. 2009 Jun 4;113(23):5951-60.
28. Flotho C, Valcamonica S, Mach-Pascual S, Schmahl G, Corral L, Ritterbach J, et al. RAS mutations and clonality analysis in children with juvenile myelomonocytic leukemia (JMML). Leukemia. 1999 Jan;13(1):32-7.
29. Van Tendeloo VF, Van de Velde A, Van Driessche A, Cools N, Anguille S, Ladell K, et al. Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proc Natl Acad Sci U S A. 2010 Aug 3;107(31):13824-9.
30. Bergmann L, Miething C, Maurer U, Brieger J, Karakas T, Weidmann E, et al. High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. Blood. 1997 Aug 1;90(3):1217-25.
31. Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, et al. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood. 1994 Nov 1;84(9):3071-9.
32. Jacobsohn DA, Tse WT, Chaleff S, Rademaker A, Duerst R, Olszewski M, et al. High WT1 gene expression before haematopoietic stem cell transplant in children with acute myeloid leukaemia predicts poor event-free survival. Br J Haematol. 2009 Sep;146(6):669-74.
33. Lapillonne H, Renneville A, Auvrignon A, Flamant C, Blaise A, Perot C, et al. High WT1 expression after induction therapy predicts high risk of relapse and death in pediatric acute myeloid leukemia. J Clin Oncol. 2006 Apr 1;24(10):1507-15.
34. Noronha SA, Farrar JE, Alonzo TA, Gerbing RB, Lacayo NJ, Dahl GV, et al. WT1 expression at diagnosis does not predict survival in pediatric AML: a report from the Children's Oncology Group. Pediatr Blood Cancer. 2009 Dec;53(6):1136-9.
35. Rodrigues PC, Oliveira SN, Viana MB, Matsuda EI, Nowill AE, Brandalise SR, et al. Prognostic significance of WT1 gene expression in pediatric acute myeloid leukemia. Pediatr Blood Cancer. 2007 Aug;49(2):133-8.
36. Spanaki A, Linardakis E, Perdikogianni C, Stiakaki E, Morotti A, Cilloni D, et al. Quantitative assessment of WT1 expression in diagnosis of childhood acute leukemia. Leukemia research. 2007 Apr;31(4):570-2.
37. Trka J, Kalinova M, Hrusak O, Zuna J, Krejci O, Madzo J, et al. Real-time quantitative PCR detection of WT1 gene expression in children with AML: prognostic significance, correlation with disease status and residual disease detection by flow cytometry. Leukemia. 2002 Jul;16(7):1381-9.
38. Ho PA, Zeng R, Alonzo TA, Gerbing RB, Miller KL, Pollard JA, et al. Prevalence and prognostic implications of WT1 mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. Blood. 2010 Aug 5;116(5):702-10.
39. Maurer U, Jehan F, Englert C, Hubinger G, Weidmann E, DeLuca HF, et al. The Wilms' tumor gene product (WT1) modulates the response to 1,25-dihydroxyvitamin D3 by induction of the vitamin D receptor. J Biol Chem. 2001 Feb 9;276(6):3727-32.
40. Maurer U, Weidmann E, Karakas T, Hoelzer D, Bergmann L. Wilms tumor gene (wt1) mRNA is equally expressed in blast cells from acute myeloid leukemia and normal CD34+ progenitors. Blood. 1997 Nov 15;90(10):4230-2.
41. Hosen N, Shirakata T, Nishida S, Yanagihara M, Tsuboi A, Kawakami M, et al. The Wilms' tumor gene WT1-GFP knock-in mouse reveals the dynamic regulation of WT1 expression in normal and leukemic hematopoiesis. Leukemia. 2007 Aug;21(8):1783-91.
42. Inoue K, Tamaki H, Ogawa H, Oka Y, Soma T, Tatekawa T, et al. Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. Blood. 1998 Apr 15;91(8):2969-76.
43. Tsuboi A, Oka Y, Ogawa H, Elisseeva OA, Tamaki H, Oji Y, et al. Constitutive expression of the Wilms' tumor gene WT1 inhibits the differentiation of myeloid progenitor cells but promotes their proliferation in response to granulocyte-colony stimulating factor (G-CSF). Leukemia research. 1999 May;23(5):499-505.



5

HLA-IDENTICAL UMBILICAL CORD BLOOD TRANSPLANTATION FROM A SIBLING DONOR IN JUVENILE MYELOMONOCYTIC LEUKEMIA

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Illustration: JMML, monocytosis and abnormal monocytes (PB)

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Prof. Dr. P.D. Emanuel for his expert advise on the management of patient 1.
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ABSTRACT

Juvenile myelomonocytic leukemia (JMML) is a rare malignant myeloid disorder in childhood, for which stem cell transplantation is currently the only curative treatment option. Although in JMML successful unrelated donor umbilical cord blood transplantations (UCBTs) have been described, series of HLA-identical sibling donor UCBTs are not available.

From the European Working Group of MDS and JMML in Childhood (EWOG-MDS) we describe 5 JMML patients who received fully HLA matched sibling UCB. All patients engrafted slowly and did not suffer from graft-versus-host-disease. These cases illustrate that relatively immunologically naive HLA-matched sibling UCBT is feasible in selected cases of JMML.

INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) is a rare type of childhood leukemia, which is characterized by young age, hepatosplenomegaly, thrombocytopenia, monocytosis, molecular aberrations in the RAS-RAF-MEK-ERK signaling pathway and GM-CSF-hypersensitivity (1-3). Although outstanding progress has been made in unraveling the molecular background of JMML, the only curative treatment option for these young children is stem cell transplantation (SCT) (1, 4-7). Previously published data have shown that in JMML the graft-versus-leukemia (GvL) effect of the SCT plays an important role in the prevention of relapse (5, 8). Hence, donor and stem cell source selection could play an important role in the outcome of an individual JMML patient. SCT using unrelated, immunological naive, umbilical cord blood (UCB) has shown to be effective for pediatric JMML (7). However, it is conceivable that the relatively immunological naivety of cord blood stem cells from a newborn HLA identical sibling donor may be a negative factor for outcome (8-9).

In this report we describe 5 JMML patients registered in the database of the European Working Group of MDS and JMML in Childhood (EWOG-MDS), who have received umbilical cord stem cells from a HLA identical sibling and we reviewed the available literature.

METHODS

For this study 5 well-documented patients who received umbilical cord stem cells from an HLA identical sibling were identified in the database of EWOG-MDS. Clinical, laboratory and survival data were retrieved from the database and from the local hospitals and all available literature was reviewed.

RESULTS

Patients: In the database of EWOG-MDS 3 Dutch, 1 Czech and 1 Austrian JMML patients, given allogeneic cord blood transplantation from a HLA-identical sibling were identified. The median age at diagnosis was 18 months (range: 15-30 months) (Table 1).

Four patients received treatment prior to the hematopoietic stem cell transplantation because of progression of disease: i.e. 3 patients received 6-mercaptopurine, 3 underwent a splenectomy prior to transplantation and 1 was treated with interferon alpha-2.

Transplantation: The median time from diagnosis to transplantation was 9 months (range 4 -12 months). The transplantation and conditioning characteristics are described in Table 2. Four patients received a conditioning regimen consisting of busulphan, cyclophosphamide and melphalan according to EWOG-MDS guidelines (10). One patient (patient 3) received cyclophosphamide, etoposide and total body irradiation. The median amount of transplanted cell dose was 0.5 x 10^8/kg body weight (range 0,16-1 x 10^8/kg body weight). All patients but one (patient 5) received cyclosporine (2-5 mg/kg/day) as graft-versus-host-disease (GVHD) prophylaxis.

Engraftment: All patients engrafted. Myeloid engraftment (ANC > 500/ul) was reached at a median of 33 days (range 10-35 days) after SCT. Platelet count above 20.000/ul was reached at a median of 48 days (range 33-52 days) after SCT.

Graft-versus-host-disease: Two patients did not show any sign of acute GVHD (patient 1 and 2). In the three other patients mild acute GVHD was observed (patient 3 grade

Table 1: Patient characteristics of the JMML patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age at diagnosis (months)	18	18	27	14	30
Sex	F	F	F	M	M
Peripheral blood counts at diagnosis					
- Hb (mmol/l)	4.5	6.8	7.5	4.8	3.6
- WBC (x 10^9/l)	68	4.6	116	20.9	11
- % blasts	4	0	0	1	13
- % monocytes	24	35	14	5	23
- PLT (x 10^9/l)	70	14	115	23	64
- %HbF	2.5	1.3	64.3	0.6	18
Bone marrow (% blasts)	6	2	3	11	7
GM-CSF hypersensitivity	yes	no	n.a.	no	n.a.
NF1/PTPN11/RAS gene mutation	no	no	n.a.	no	*PTPN11*
Karyotype	46, XX	46, XX	46, XX	45, XY, -7	45, XY, -7
Treatment prior to transplantation	6-MP	none	interferon α-2, splenectomy	6-MP splenectomy	6-MP splenectomy

Hb = haemoglobin
WBC = white blood cell
Plt = platelets
GM-CSF = granulocyte-macrophage colony stimulating factor
M = male
F = female
n.a. = not available

1, patient 4 and 5 grade 2). No chronic GVHD was reported. The immunosuppressive therapy was discontinued at a median of 33 days (range 0 - 87 days) after SCT.

Relapse: Two patients (patient 3 and 5) relapsed 6 months and 30 months after SCT respectively. In both patients a second transplantation procedure with bone marrow of the initial donor was performed. One patient is in second complete remission (patient 5). The other patient died after a second relapse (Table 2).

Chimerism: In all patients chimerism analysis was performed and after transplantation full donor chimerism was found in all cases. In the cases that relapsed a mixed chimerism was found prior to clinical progression. In addition, in one case (patient 1) increasing mixed donor chimerism developed from day 42 on, despite 2 donor lymphocyte infusions (DLI) with no clinical signs of relapse (Figure 1). Second stem cell transplantation was considered, in order to avoid relapse. The parents however, refused the procedure because of the absence of any clinical signs of JMML and the excellent clinical condition of the child. Therefore, ultimately, 6-mercaptopurine (6-MP) (average dose 40 mg/m2) was started on day 145 after SCT (Figure 1). When, after initial improvement, donor chimerism increased, 6-MP was replaced by 13-cis-retinoic acid (100 mg/m^2/day every

Table 2: Transplant characteristics and outcome.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
HLA-match	10/10 identical	10/10 identical	10/10 identical	10/10 identical	10/10 identical
Cell dose (X 10^8 MNC/kg)	1	n.a.	0.2	0.4	0.5
Conditioning:					
Cy/etoposide /TBI					
- Total Body Irradiation			12 Gy		
- Methotrexate intrathecal			2x 12 mg		
- Day -4: etoposide (iv)			60 mg/kg		
- day -3- -2: cyclophosphamide (iv)			60 mg/kg		
Bu/Cy/Mel					
- Day -9- -6: busulphan (iv/ oral)	4-6mg/kg	120 mg/m2		5 mg/kg	120 mg/m2 oral
- Day -4 - -3:cyclophosphamide (iv)	60 mg/kg	60 mg/kg		60 mg/kg	60 mg/kg
- Day -1: melphalan (iv)	140 mg/m2	140 mg/m2		140 mg/m2	140 mg/m2
GVHD-prophylaxis:					
- cyclosporine	2 mg/kg	2 mg/kg	5 mg/kg	3 mg/kg	none
Tapering GVHD prophylaxis	day 28	day 42	n.a.	n.a	-
Stop GVHD prophylaxis	day 42	day 70	day 87	day 7	-
Therapy after transplantation	2x donor lymphocytes	no	re-transplantation	no	5x donor lymphocytes re-transplantation
Relapse	no	no	yes	no	yes
Time till first relapse (months)			4 months		30 months
Survival from (last) SCT	alive	alive	death	alive	alive
Follow up time (months)	60	18		84	66

TBI = total body irradiation
GVHD = graft versus host disease
SCT = stem cell transplantation
G-CSF = granulocyte colony stimulating factor
n.a = not available

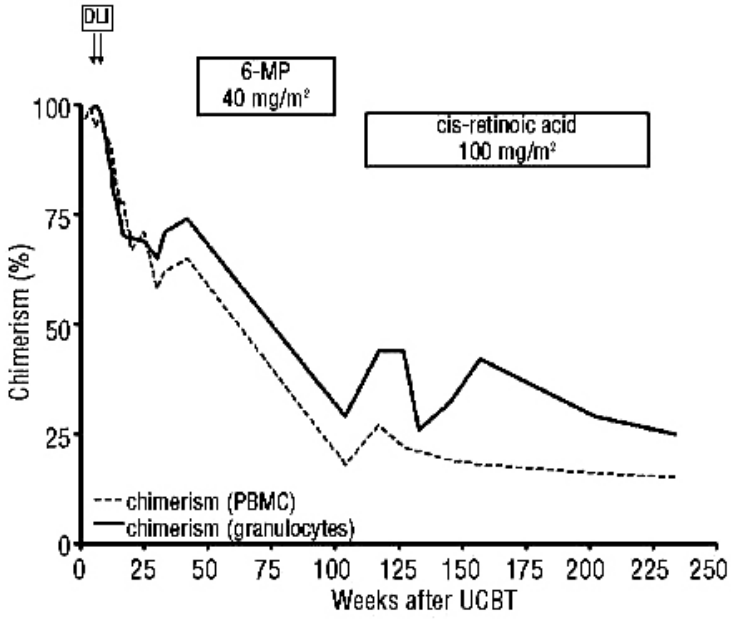


Figure 1: Chimerism data of patient 1.
DLI = donor lymphocyte infusion (week 10 and 12)
6-MP = 6- mercaptopurine
PBMC = peripheral blood mononuclear cells
UCBT = umbilical cord blood transplantation
% chimerism reflects the percentage of donor bone marrow in the patient

other week) for 2.5 years until the age of 5 years. Thereafter, the child remained well and alive without any signs of JMML with a current follow-up of 5 years after SCT. At present the percentage donor cells is 15%, without any clinical sign of JMML.

Survival: Four of five patients are in complete remission after a HLA identical sibling cord blood transplantation, one of them after a second transplantation (follow up: 1½ till 7 years after transplantation). One patient died due to a relapse after second transplantation.

DISCUSSION

In 1989 Gluckman *et al* described the first successful umbilical cord blood transplantation (UBCT) from an HLA-identical sibling in a child with Fanconi anemia (11). The patient is currently alive and free of disease (12). UBCT has increased the available pool of hematopoietic stem cell transplantation donors, especially for young children, in which the UCB stem cell yield is usually sufficient without further *ex vivo* expansion (13). This is important for patients with rare HLA-haplotypes and for patients with diseases that may rapidly progress like JMML. Moreover, the lower risk of infection transmission by using UCB may be of benefit (5, 7-8).

So far only 1 study reported on sibling UCBT in JMML. MacMillan *et al* described the results of UCBT in 3 children with JMML, one of which received HLA identical sibling umbilical cord stem cells. This boy, 3 months old at diagnosis, received 1.4 x 10^8/kg nucleated cells from his sister after conditioning with cyclophosphamide (60 mg/kg x 2d) and total body irradiation (165 cGy bid x 4d). On day 60 after transplantation complete donor chimerism was observed. He suffered from grade 1 acute GVHD, but no signs of chronic GVHD were observed. He relapsed at day 95 and a second transplantation with the same donor was performed on day 234 after the first transplantation. On day 42 after the second transplantation complete donorchimerism was found. Nevertheless, he relapsed again at day 101 after the second transplantation after which he died (14).

More published data are available on the use of unrelated UCBT in JMML. Tanoshima *et al* reported a girl with JMML who received an unrelated matched UCBT. She developed mild acute GVHD of the skin and grade 3 acute GVHD of the gastrointestinal tract. Cyclosporine was discontinued at day 103. Thereafter, no signs of GVHD occurred and she remained in complete remission, with full donor chimerism at a follow up of 1 year after transplantation (15). MacMillan *et al* described 2 children with JMML receiving an unrelated UCBT. The first child received 1.04 x 10^8 HLA-A, -B, -DR1 matched nucleated cells/kg after conditioning with cyclophosphamide (60 mg/kg x 2d) and TBI (165 cGy b.i.d. x 4d). She suffered from acute GVHD (grade 3) and extensive chronic GVHD. Two years after SCT complete donor chimerism and no signs of relapse were reported. The second boy received 0.38 x 10^8 nucleated cells/kg from a donor with a HLA- B and -DRB1 mismatch. The conditioning consisted of cyclophosphamide (60 mg/kg x 2d) and TBI (165 cGy b.i.d. x 4). This child never achieved complete donor chimerism and at day 65 after transplantation he relapsed. Subsequently, he was treated with chemotherapy (cytarabine and etoposide) and on day 125 after transplantation he was alive with disease (14). From the combined EWOG-MDS/EBMT registry Locatelli *et al* described 100 JMML patients of which 7 received an unrelated UCBT. These 7 patients showed a delayed hematologic recovery, but the outcome was comparable to the children treated with other stem cell sources (HSCT) (7). In non-JMML pediatric patients, related UCBTs have been reported in a cohort of 190 children with malignancies, bone marrow failure syndromes, hemoglobinopathies and inborn errors of metabolism or primary immunodeficiencies. The survival rates 3 years after transplant were 47%, 82%, 100% and 70% respectively (16). In another study, Rocha *et al* described the occurrence of GVHD in children who received related UCBT (n=113) or bone marrow transplantation (BMT) from HLA identical siblings (n=2052). Patients receiving related UCBT showed a slower hematopoietic recovery (median 28 days versus 18 days for neutrophil count ≥ 500/mm^3) and lower risk of acute and chronic GVHD as compared to the group who received related bone marrow transplantation (BMT). There was no significant difference between the two groups in relapse related death, mortality rate at day 100 and overall survival (17). Gluckman *et al* compared the outcome of patients who received a UCBT of related donors to UCBT of unrelated donors. Indications for transplantation were malignancies, bone marrow failure syndromes, hemoglobinopathies and inborn errors. Of the patients transplanted with a related donor, 18% suffered from acute GVHD of at least grade 2 compared to 32% in the unrelated donor group (18). These studies underscore the relative immune naivety of the UCB cells and the feasibility of the use

of UCBs especially for childhood transplantation settings. However, especially in JMML, where the GvL-effect is an important contributor to the success of the transplantation procedure, although our patients survived, it could be questioned whether these, if available, relatively naive umbilical stem cells from sibling donors should be used in all patients, especially when alternative donors would be available.

The fact that one of our patients showed mixed donor chimerism shortly after SCT may reflect the absence of GvL after HLA identical UCBT. Mixed donor chimerism after SCT in JMML has been shown to be an important predictor for relapse. If this occurs early after transplantation discontinuation of immunosuppressive therapy (IST) is the first step to prevent relapse as has been shown by the EWOG-MDS (19). In our patient IST has already been discontinued and the parents were very reluctant to proceed to a second SCT because of absence of clinical signs of JMML. Therefore we decided to initiate 6-MP as pre-emptive treatment, followed by cis-retinoic acid. At the moment no series are available on the use of these compounds in a relapse after SCT for JMML. Although Locatelli *et al* showed that second SCT was successful in 7/15 cases with a relapse after SCT, the issue whether to proceed to second SCT in all cases of mixed donor chimerism without any clinical signs of JMML has not been elucidated (7). Although in the past donor lymphocytes infusions have been described to be successful in individual cases, EWOG data have shown that in JMML DLIs are not usually beneficial for the prevention of relapse in cases with mixed donor chimerism (20-21).

It is uncertain which factors contributed to the favorable outcome of our patients so far. Our JMML patients were young and this has been shown to be an important prognostic factor (7). Four of our patients did not have known mutations in the RAS-RAF-MEK-ERK pathway. It is possible that their biological background thus harbours a better prognosis after HSCT, although so far no reports are available about the predictive value of the mutational status of JMML patients on outcome after transplantation.

We conclude that in JMML umbilical cord stem cells can be considered a good alternative source for HSCT. However, whether the use of sibling HLA-identical UCBs should be advocated needs to be confirmed in larger series of JMML patients.

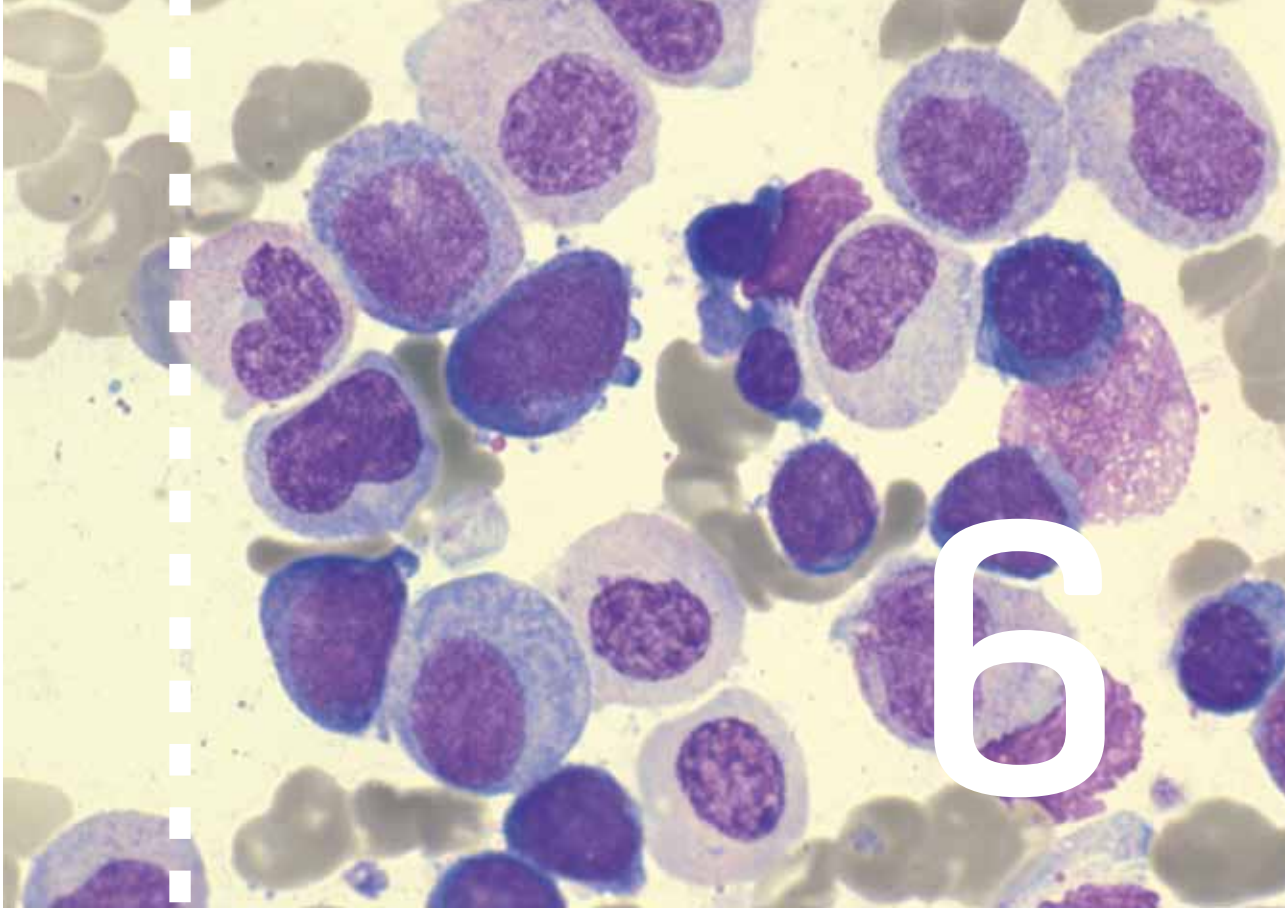
REFERENCES

1. Side LE, Emanuel PD, Taylor B, Franklin J, Thompson P, Castleberry RP, et al. Mutations of the NF1 gene in children with juvenile myelomonocytic leukemia without clinical evidence of neurofibromatosis, type 1. Blood. 1998 Jul 1;92(1):267-72.
2. Niemeyer CM, Arico M, Basso G, Biondi A, Cantu Rajnoldi A, Creutzig U, et al. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). Blood. 1997 May 15;89(10):3534-43.
3. Niemeyer CM, Fenu S, Hasle H, Mann G, Stary J, van Wering E. Differentiating juvenile myelomonocytic leukemia from infectious disease (letter). Blood. 1998;91(1):365-7.
4. Flotho C, Valcamonica S, Mach-Pascual S, Schmahl G, Corral L, Ritterbach J, et al. RAS mutations and clonality analysis in children with juvenile myelomonocytic leukemia (JMML). Leukemia. 1999 Jan;13(1):32-7.
5. Locatelli F, Niemeyer C, Angelucci E, Bender-Gotze C, Burdach S, Ebell W, et al. Allogeneic bone marrow transplantation for chronic myelomonocytic leukemia in childhood: a report from the European Working Group on Myelodysplastic Syndrome in Childhood. J Clin Oncol. 1997 Feb;15(2):566-73.
6. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J, Jung A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat Genet. 2003 Jun;34(2):148-50.
7. Locatelli F, Nollke P, Zecca M, Korthof E, Lanino E, Peters C, et al. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): results of the EWOG-MDS/EBMT trial. Blood. 2005 Jan 1;105(1):410-9.

8. Smith FO, King R, Nelson G, Wagner JE, Robertson KA, Sanders JE, et al. Unrelated donor bone marrow transplantation for children with juvenile myelomonocytic leukaemia. Br J Haematol. 2002 Mar;116(3):716-24.
9. Manabe A, Okamura J, Yumura-Yagi K, Akiyama Y, Sako M, Uchiyama H, et al. Allogeneic hematopoietic stem cell transplantation for 27 children with juvenile myelomonocytic leukemia diagnosed based on the criteria of the International JMML Working Group. Leukemia. 2002 Apr;16(4):645-9.
10. Locatelli F, Pession A, Comoli P, Bonetti F, Giorgiani G, Zecca M, et al. Role of allogeneic bone marrow transplantation from an HLA-identical sibling or a matched unrelated donor in the treatment of children with juvenile chronic myeloid leukaemia. Br J Haematol. 1996 Jan;92(1):49-54.
11. Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med. 1989 Oct 26;321(17):1174-8.
12. Gluckman E, Rocha V. History of the clinical use of umbilical cord blood hematopoietic cells. Cytotherapy. 2005;7(3):219-27.
13. Barker JN, Krepski TP, DeFor TE, Davies SM, Wagner JE, Weisdorf DJ. Searching for unrelated donor hematopoietic stem cells: availability and speed of umbilical cord blood versus bone marrow. Biol Blood Marrow Transplant. 2002;8(5):257-60.
14. MacMillan ML, Davies SM, Orchard PJ, Ramsay NK, Wagner JE. Haemopoietic cell transplantation in children with juvenile myelomonocytic leukaemia. Br J Haematol. 1998 Nov;103(2):552-8.
15. Tanoshima R, Goto H, Yanagimachi M, Kajiwara R, Kuroki F, Yokota S. Graft versus leukemia effect against juvenile myelomonocytic leukemia after unrelated cord blood transplantation. Pediatr Blood Cancer. 2007 Apr 16.
16. Rocha V, Gluckman E. Clinical use of umbilical cord blood hematopoietic stem cells. Biol Blood Marrow Transplant. 2006 Jan;12(1 Suppl 1):34-41.
17. Rocha V, Wagner JE, Jr., Sobocinski KA, Klein JP, Zhang MJ, Horowitz MM, et al. Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. N Engl J Med. 2000 Jun 22;342(25):1846-54.
18. Gluckman E, Rocha V, Boyer-Chammard A, Locatelli F, Arcese W, Pasquini R, et al. Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. N Engl J Med. 1997 Aug 7;337(6):373-81.
19. Yoshimi A, Niemeyer CM, Bohmer V, Duffner U, Strahm B, Kreyenberg H, et al. Chimaerism analyses and subsequent immunological intervention after stem cell transplantation in patients with juvenile myelomonocytic leukaemia. Br J Haematol. 2005 May;129(4):542-9.
20. Worth A, Rao K, Webb D, Chessells J, Passmore J, Veys P. Successful treatment of juvenile myelomonocytic leukemia relapsing after stem cell transplantation using donor lymphocyte infusion. Blood. 2003 Mar 1;101(5):1713-4.
21. Yoshimi A, Bader P, Matthes-Martin S, Stary J, Sedlacek P, Duffner U, et al. Donor leukocyte infusion after hematopoietic stem cell transplantation in patients with juvenile myelomonocytic leukemia. Leukemia. 2005 Jun;19(6):971-7.

PART TWO

MYELODYSPLASTIC SYNDROME IN CHILDHOOD



6

MOLECULAR ABERRATIONS IN 107 CHILDREN WITH MYELODYSPLASTIC SYNDROME (MDS)

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Submitted

Illustration: MDS, hypogranulation and nuclear-cytoplasm-asynchrony in myelopoiesis (BM)

ABSTRACT

Background: The integrative non-random collaboration of type II and type I mutations which induce maturation arrest and hyperproliferation respectively, has been extensively studied in myeloid malignancies. So far, information on the involvement of these aberrations in pediatric myelodysplastic syndrome (MDS) is lacking.

Design and methods: We studied the occurrence of genetic aberrations in an international cohort of pediatric MDS patients (n=44 primary MDS, n=63 secondary MDS). Karyotypes were studied and the hotspot regions of the *NPM1, CEPBA, FLT3, NRAS, KRAS, BRAF, PTPN11, c-KIT, RUNX1, P53, ASXL1, NUP98, IDH1, IDH2, DNMT3A* and *TET2* genes were screened for mutations.

Results: Type I aberrations were found in 8/107 (7.5%) patients (4/44 primary MDS, 4/63 secondary MDS): mutations in *FLT3–ITD* (n=3), *NRAS* (n=2), *KRAS* (n=2) and *PTPN11* (n=1). No mutations were found in the *FLT3-TKD*, *c-KIT*, *P53* and *BRAF* gene. Known Type II aberrations were identified in 17/107 (16%) (4/44 pMDS, 13/63 sMDS): *MLL*-rearrangements (n=2), *RUNX1*-rearrangement (n=1), *RUNX1* mutations (n=7), duplications in the *CEBPA* gene (n=5), *NPM1* gene mutation (n=1) and *NUP98* translocation (n=1). We also identified *ASXL1* mutations (n=2) and *DNMT3A* mutations (n=1), but no *TET2*, *IDH1* and *IDH2* mutations were found in any of the pediatric MDS cases. Only two sMDS patients carried both a type I and a type II mutation (*NRAS* + *RUNX1* mutation and *ASXL1* + *RUNX1* mutation).

Conclusions: In 9% of the primary MDS cases a type I aberration was found and in 9% a type II aberration, all being mutually exclusive. In secondary MDS type I aberrations occurred in 6% and type II aberrations in 24% of the cases (including the mutations in genes regulating the histone function and DNA methylation). In only 2% of all cases (sMDS only) a collaborative type I and II mutation was found.

This study indicates that in childhood MDS, in contrast to adult MDS, the currently known molecular aberrations, especially the histone modificating and DNA methylating influencing, are of minor importance in the pathogenesis of childhood MDS.

INTRODUCTION

Myelodysplastic syndrome (MDS) is a clonal stem cell disorder (1). MDS is rare in childhood, accounting for less than 5% of all hematological malignancies (2-3). Until now, only scarce information is available on the pathogenesis of pediatric MDS.

Kelly and Gilliland hypothesized that the development of myeloid malignancies requires at least two types of genetic events, i.e. type II aberrations leading to impaired differentiation of the leukemic cells, and type I aberrations which are involved in the proliferation of leukemic cells. In adults MDS it is generally accepted that, like in acute myeloid leukemia (AML), a multistep process involving interaction between type I and II aberrations is involved (4-5).

In therapy-related adult MDS, type I aberrations were found in 24% of the cases and type II aberrations in 34% and the coexistence of a type I and II aberration was found in 13% (5), whereas in primary adult MDS in 14% of the cases at least two gene mutations and in 57% at least one gene mutation were found (6). Several studies described the occurrence of single mutations in primary MDS. *MLL*-rearrangement, *FLT3-TKD* and mutations in *FLT3-ITD, NRAS, KRAS, PTPN11, c-KIT, NPM1* and *CEBPA* have all been found in 0-6% of the cases (6-14). Recently, *RUNX1*-rearrangements, and mutations in *RUNX1, p53, TET2, ASXL1, DNMT3A, IDH1, IDH2* and *EZH2* (9, 15-28) have been described in adult MDS in percentages ranging from 0-26%, illustrating the predominant contribution of mutations in the genes regulating the histone function and DNA methylation in adult MDS. Furthermore, in adult MDS it is suggested that the RNA splicing machinery is one of the most affected pathways with a mutation rate of 35-85% (29-30).

To date, it is unknown whether type I and II aberrations contribute to the pathogenesis of pediatric MDS. Therefore, we analysed an international cohort of primary and secondary childhood MDS cases for the occurrence of these types of aberrations.

PATIENTS & METHODS

Study cohort: We included 107 pediatric MDS patients (n=44 primary MDS, and n=63 secondary MDS). Secondary MDS was defined as MDS occurring after treatment with chemo- and/or radiotherapy (n=39) or after an initial diagnosis of bone marrow failure syndrome (n=24). Five familial MDS cases were included in the primary MDS cohort. Samples and clinical and cell-biological data, including standard cytogenetics were provided by the centers participating into the European Working Group of MDS and JMML in Childhood (EWOG-MDS) and from one center from Greece (n=2 familial cases). Informed consent was obtained from patient's parents or their legal guardians according to local laws and regulations (31). Each country performed central review of the bone marrow morphology, according to the WHO classification, and the 2 Greek familial cases were included in this pathology review (32-33).

DNA and RNA isolation: After thawing of the available viably frozen bone marrow or peripheral blood samples, malignant cells were isolated as previously described (34). Genomic DNA and total RNA were extracted from mononuclear cells using TRIzol reagent (Invitrogen Life Technologies, Breda, The Netherlands) according to manufacturer's instructions (35). To perform all the mutation analyses, we amplified the original DNA using the Bioscore screening and amplification kit (Enzo Life Science, Harmingdale, NY,

United States) according to the manufacturer's instructions. When an aberration was found using the amplified material, the aberration always was confirmed on the original non-amplified material.

Cytogenetic analysis: MDS samples were routinely investigated for cytogenetic aberrations by G-, Q-, or R-banded karyotyping, by the national group for AML and MDS specific translocations including inv(16)(p13q22)/t(16;16)(p13;q22), t(8;21)(q22;q22), t(15;17)(q24;q21) and *EVI1*-rearrangement (36-37). Furthermore, in situ hybridization (FISH) for identification of monosomy 7 and trisomy 8 was performed when conventional cytogenetics failed (36). Furthermore, complexicity of the karyotype was defined as described by Gohring et al (38).

Molecular aberrations: For this study mutations were determined in the hotspot regions of FLT3 (i.e. internal tandem duplications *(ITD)* and *FLT3* tyrosine kinase domain *(TKD)* point mutations), *NRAS, KRAS, NPM1, CEPBA, PTPN11* and *C-KIT*, as previously described (39-47). In addition, we screened for mutations in exon 13 of the *ASXL1* gene, exon 5-8 of the *P53* gene, exon 3-8 of the *RUNX1* gene, the whole *TET2* gene, exon 4 of both the *IDH1* and *IDH2* gene and *DNMT3A* on cDNA (21, 48). A complete list of screened regions per gene, primers used and PCR conditions is provided in Table 1. The fusion genes including *MLL*-rearrangements, *EVI1*-rearrangements, *PML-RARα, NUP98-NSD1* fusion and mutations in the *NPM1, CEBPA, ASXL1, TET2, IDH1* and 2, *DNMT3A* and RUNX1 gene were considered as type II aberrations whereas mutations in *FLT3*, N*KRAS, PTPN11* and *C-KIT* were considered to be type I aberrations, as previously discussed (47-49).

Statistical analysis: Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, Ill, USA). Different variables were compared with the Chi-square/Fischer exact test or the Mann-Whitney-U test. P-values <0.05 were considered significant (2-tailed).

RESULTS

Study cohort: Forty-four primary MDS and sixtythree secondary MDS patients were included for this study. Median age was 9.1 years for the primary MDS (range: 0-18 years) and 13.1 years for the secondary MDS (range: 1.2-26 years). Based on morphology, 17 primary MDS patients were diagnosed as refractory cytopenia and 27 as advanced MDS. Of the secondary MDS cases, 27 cases were diagnosed as refractory cytopenia and 36 secondary MDS patients as advanced MDS. Table 2 reports the clinical data and details of the cytogenetic aberrations of those patients with a type I or II aberration.

Type I aberrations

The success rate of the mutation analysis ranged from 96-100% for the various aberrations. In total, 8/107 type I aberrations were found, i.e 3 *FLT3-ITD*, 2 *NRAS* mutations (G12A), 2 *KRAS* (G11L) and one *PTPN11* (A72V). No mutations were found in the *FLT3-TKD, c-KIT, BRAF* and *P53* gene in any of the MDS patients at diagnosis. In 6 cases a SNP was found in exon 17 in the *c-KIT* gene (I797I). No SNPs were identified in the *RAS, BRAF, PTPN11* and *P53* genes.

Table 1: Hotspot regions and primers used for mutation analysis

gene	Primer
ASXL1_A	F: 5'- GCT ACA GAA TCC TAG TTT TGC TTT A-3' R: 5'- TTT GTC ACT GCA GCT TCT CTA-3'
ASXL1_B	F: 5'- TGG ACA TTG AAA AGC TGA AA-3' R: 5'- CCT GCC TAA AGA GTA TTA TAT CAA TG-3'
ASXL1_A1	F: 5'- GCT ACT AGA ATC CTA GTT TTG CTT TA-3' R: 5'- GCA GTA GTT GTG TTC GCT GT-3'
ASXL1_A2	F: 5'- AGC AGC AGT GGT GAT GG-3' R: 5'- GGA ACT GGG GGT CAC AT-3'
ASXL1_A3	F: 5'- TGT CTC TAG TGG GAG ATG ATA CA-3' R: 5'- TTT GTC ACT GCA GCT TCT CTA-3'
ASXL1_B1	F: 5'- TGG ACA TTG AAA AGC TGA AA-3' R: 5'- TTC ACA GCT ATC TGG CAG AA-3'
ASXL1_B2	F: 5'- GAA TCC CCA CAA GTA CAC T-3' R: 5'- GGA CCA AAG GAG ATC ACA TT-3'
ASXL1_B3	F: 5'- GGA TTC CAA AGA GCA GTT CT-3' R: 5'- TCG GGG TAA TTT CCA GAA-3'
ASXL1_B4	F: 5'- CAG CGT CAA GAA TGA GAA GA-3' R: 5'- CCT GCC TAA AGA GTA TTA TAT CAA TG-3'
BRAF	F: 5'- AGC CCC AAA AAT CTT AAA AG-3' R: 5'- CTC AGG GCC AAA AAT TTA AT- 3'
CEBPA	F: 5'- CGC CAT GCC GGG AGA ACT CT-3' R: 5'- CTT GGC TTC ATC CTC CTC GC-3' F: 5'- CGG CCG CTG GTG ATC AAG-3' R: 5'- CCA GGG CGG TCC CAC AGC-3'
c-KIT (exon 8)	F: 5'- CCG CCT CCT TGT ACC TT-3' R: 5'- TTC AGC AAA CAA AAT TAA TGT CTA-3'
c-KIT (exon 17)	F: 5'- TCC TCC AAC CTA ATA GTG TAT TC-3' R: 5'- CAT TCC GAA ATC AAA CAG TT-3'
DNMT3A (exon 12-23)	F: 5'- GCC ACC AGA AGA AGA GAA GA-3' R: 5'- GCA CCT GCA GCA GTT GT-3' F: 5'- CCG GAA CAT TGA GGA CA-3' R: 5'- CTG GGA CAG GTG GGT AAA-3' F: 5'- CGG AGG TGT GTG TGA GGA CT-3' R: 5'- GTT CAT ACC GGG AAG GTT AC-3' F: 5' - CTC CAG ATG TTC TTC TTC GCT AA-3' R: 5'- CCA CTC CTG GAT ATG CTT CT-3'
FLT3_ITD	F: 5'- GCA ATT TAG GTA TGA AAG GCC AGC-3' R: 5'- CTT TCA GCA TTT TGA CGG CAA CC-3'
FLT3_TKD	F: 5'- TCA CCG GTA CCT CCT ACT TG-3' R: 5'- AAA TGC ACC ACAGTG AGT G-3'
NPM1	F: 5'- CTG GTG GTA GAA TGA AAA ATA GAT-3' R: 5'- GGC AGGACA TTC TCA TAG-3'
NUP98	F: 5' - TGG ACA GGC ATC TTT GTT -3' R: 5'- ACA GCG GGA ACT TAC CTT-3'
PTPN11 (exon 3)	F: 5'- TTG GGT TTC TTT CAA CAC TT-3' R: 5'- GCC TTT GGA GTC AGA GAG T-3'

Table 1: Hotspot regions and primers used for mutation analysis

gene	Primer
PTPN11 (exon 13)	F: 5'- TGG CTC TGC AGT TTC TCT-3' R: 5'- CAT TCC GAA ATC AAA CAG TT-3'
P53 (exon 5)	F: 5'- CCG TCT TCC AGT TGC TTT-3' R: 5'- CAA CCA GCC CTG TCG T-3'
P53 (exon 6)	F: 5'- GCA CAT GAC GGA GGT TG-3' R: 5'- AGG AGA AAG CCC CCC TA-3'
P53 (exon 7)	F: 5'- CGG TGG AGC TTG CAG T-3' R: 5'- CCG GGG ATG TGA TGA G-3'
P53 (exon 8)	F: 5'- GGT TTT TTA AAT GGG ACA GG-3' R: 5'- TAG GAA AGA GGC AAG GAA AG-3'
KRAS (exon 1)	F: 5'- CGT CGA TGG AGG AGT TT-3' R: 5'- AAC CCA AGG TAC ATT TCA GA-3'
NRAS (exon 1)	F: 5'- GGG GGT TGC TAG AAA ACT A-3' R: 5'- ATC CGA CAA GTG AGA GAC A-3'
NRAS (exon 2)	F: 5'- CCC AGG ATT CTT ACA GAA AA-3' R: 5'- TCC CCA TAA AGA TTC AGA AC-3'
RUNX1 (exon 3)	F: 5'-AGCTGTTTGCAGGGTCCTAA-3' R: 5'-GTCCTCCCACCACCCTCT-3'
RUNX1 (exon 4)	F: 5'- CATTGCTATTCCTCTGCAACC-3' R: 5'- CCATGAAACGTGTTTCAAGC-3'
RUNX1 (exon 5)	F: 5'- CCACCAACCTCATTCTGTTT-3' R: 5'- AGACATGGTCCCTGAGTATA-3'
RUNX1 (exon 6)	F: 5'- GGGGGCCCATTCTGCTGAGAGG-3' R: 5'- GAGCATCAAGGGGAAACCCC-3'
RUNX1 (exon 7)	F: 5'- AATCCCACCCCACTTTACAT-3' R: 5'- CTCAGCTGCAAAGAATGTGT-3'
RUNX1 (exon 8)	F: 5'- TCCGCTCCGTTCTCTTGC-3' R: 5'- GCTTGTCGCGAACAGGAG-3'
TET2 (exon 3)	F: 5'- CAG TTT GCT ATG TCT AGG TAT CCG A-3' R: 5'- AGA AGG GTT CAC TAA CTG TGC GTT TT-3' F: 5'- TTC AAC TAG AGG GCA GCC TTG-3' R: 5'- TGTGCGTTT TAT TCC TCC ATT TT-3' F: 5'- CAG AAT AGT CGT GTG AGT CCT GAC-3' R: 5'- GCA ATG GAA ACA CAA TCT GGA-3' F: 5'- GAA CAC ACA CAT GGT GAA CTC C-3' R: 5'-AAT TGT GAT GGT GGT GGT GG-3' F: 5'- TCC AGG GAA CCA CAA AGC TAG-3' R: 5'-GCT TGA GGT GGT CTG ACA TTG G-3' F: 5'- ACA TGT ATG CAG CCC TTC TCC-3' R: 5'- GGG AAT CTG CTC TTT GTT GAA A-3' F: 5'- ACC AAC ATC TCC AGT TCC AA-3' R: 5'- ATG CAC TTG ATT TCA TGG TCT-3' F: 5'- CAA ATG GGA CTG GAG GAA GT-3' R: 5'- GTT TGC TGC TGT TCT TGC TT-3' F: 5'- CAG AAG GAC ACT CAA AAG CAT G-3' R: 5'- TTG CTG CTC TAA AGC TGG G-3' F: 5'- GAG AAT CCA CCT GCA AGC TG-3' R: 5'- TTT CAC AAG ACA CAA GCA TCG-3'

Table 1: Hotspot regions and primers used for mutation analysis

gene	Primer
TET2 (exon 4)	F: 5'- TCT AAT AGA TCA GTC CA-3' R: 5'- CAG CCT TCA CAC ACA AAG CAG-3'
TET2 (exon 5)	F: 5'- TTC ATT TCT CAG GAT GTG GTC ATA G-3' R: 5'- TGT AAA TCT GAC CCT GAG AAT TGG-3'
TET2 (exon 6)	F: 5'- GTT GCC CTA ATT GTG ATC TAA ACA TG-3' R: 5'- CCA CTG ATA GGA AAG CCC AAT CT-3'
TET2 (exon 7)	F: 5'- GGG TTC TAC TTA ACT GGG TAT TTT CCA-3' R: 5'- GTG AAC AAT ATG ACA TAT CTT GGT AAG CT-3'
TET2 (exon8)	F: 5'- GGA TTC AAA ATG TAA GGG GAA TAA TC-3' R: 5'- TCC TCT TAA TTG TTG AAA CCA CTG C-3'
TET2 (exon 9)	F: 5'- CCA TGT CAA GAT ATT TGC TCT CTA TTT TGT-3' R: 5'- GAC TCA CAG TTC CAC ATG GCT G-3'
TET2 (exon 10)	F: 5'- CCA CCA ACA CAA ATC TGA ATA CTG A-3' R: 5'- GGT TTT GCC CCC ATC AAC TT-3'
TET2 (exon 11-1)	F: 5'- TCT TTG CTT AAT GGG TGT CGT ATA TC-3' R: 5'- GGC TTT TGA ATC AGA ATA CCC AA-3'
TET2 (exon 11-2)	F: 5'- CCA GCC CTA TGA ACT TCT ATT C-3' R: 5'- CCA TAA CTA CAG TGC AGC TCC G-3'
TET2 (exon 11-3)	F: 5'- CAA ACA TGG ACT ATA AAA ATG GTG AAC-3' F: 5'- CAC CAG GAT CTC CCT CGT CTT-3'
TET2 (exon 11-4)	F: 5'- GCC GTG GCT CCA ACT CAT-3' R: 5'- GGC AGT GGG AAA GGT CAC-3'

Type II aberrations

In total, 19 type II aberrations were found (n=4 pMDS, n=15 sMDS), i.e 2 *MLL*-rearrangement, 1 *RUNX1*-rearrangement, 1 *NUP98*-translocation, 1 *NPM1* mutation (c.859-860 insG), 5 *CEBPA* aberrations (c.590_595dupACCCGC no double mutants were identified), 2 *ASXL1* mutations (Q733stop, E1102D), 1 *DNMT3A* (N717H) and 7 *RUNX1* mutations (n=3 L56S, n=1 L134P, n=1 R204Q, n=1 Q264, insertion exon 8). For only 1/7 patients with a *RUNX1* mutation, germline material was available which identified *RUNX1* mutation in the germline material. For the other identified aberrations no germline material was available. *NUP98/NDS1* fusion product was not identified in any of the 60 patients screened. Silent mutations were found in the *P53* gene and *ASXL1* gene both in two patients. Interestingly, *TET2*, *IDH1* and *IDH2* mutations were not detected in any of the 107 patients. Several known SNPs in the *TET2* gene were found: P29H in 6 cases, L34F in 4 cases, V218M in 6 cases, G355D in 4 cases, P363L in 7 cases, M1701I in 4 cases, V1718L in 2 cases, L1721W in 23 cases, P1722S in 2 cases, I1762V in 63 cases and H1778R in 3 cases. In 25 patients a previously described SNP was found in the *CEBPA* gene (T230T). In 5 cases a known SNP was found in the *IDH1* gene (rs59682327).

So, overall of the 44 primary MDS patients 4 patients carried a type I aberration (9%) and 4 patients carried a type II aberration (9%). The mutations were mutually exclusive. Of the secondary MDS patients 4/63 (6%) had a type I aberration and 15/63 (24%) a type II aberration. Only two patients carried both a type I and a type II aberration, i.e. a

Table 2: Clinical Characteristics and Cytogenetic data of 25 MDS patients with type I and/or type II aberrations

Patient	Age at diagnosis	Gender	WHO	Blasts(%)	Cytogenetics	Mutation found	Rearrangements
1	8	F	RAEB	18	na	FLT3 ITD	
2	18	M	sRAEB-t	29	46,XY,add(2)(q24),add(7)(q31)[14]/46,XY[1]	FLT3 ITD	
3	15	F	RAEB	11	47,XX,+1,der(1;7)(q10;p10),+11[22]	FLT3 ITD	
4	14	M	sRAEB	15	45,XY,-7[12]/46,XY[3]	KRAS	
5	1	F	sCMML	5	46,XX	KRAS	
6	7	M	RAEB	15	46,XX,t(6;9)(p23;q34)[14]	NRAS	
7	15	F	sRCC	na	45,XX,-7	NRAS, RUNX1	
8	8	M	RCC	0	45,XY,ring(6)(p ?),-7[22]/46,XY[6]	PTPN11	
9	2	M	RAEB-t	27	46,XY,-?20,+mar[cp3]	CEBPA	
10	5	M	RAEB-t	21	46,XY	CEBPA	
11	3	M	sRAEB	9	45,XY,der(6)t(3;6)(q21;q15),der(17)t(17;20)(p13;q11),-20,inc	CEBPA	
12	20	M	sRAEB	7	46,XY,del(7)(q22q31)	CEBPA	
13	15	F	sRAEB	9	46,XX	CEBPA	
14	4	M	sRAEB	14	45,XY,der(14;22)(q10;q10)t(11;22)(q23;q11)c,der(18)(t(11;18)(q23;q12)		MLL-rearrangement
15	7	F	sCMML	na	46,XX,t(1;11)(p?32;q23)[16]/46,XX[4]		MLL-rearrangement
16	7	F	sRAEB-t	29	45,XX,-7[10]/46,idem,+21[5]	NPM1	
17	16	M	sRAEB	17	46,XY,inv(11)(p15q22)[13]/47,idem,+8[5]/46,idem,del(10)(p12p14)[2]		NUP98 translocation
18	19	F	sRCC	2	47,XX,del(7)(q22~31),+add(21)(q22)		RUNX1 rearrangement
19	14	F	RAEB-t	22	46,XX	RUNX1	
20	1	M	RAEB	13	45,XX,-7[10]/46,XX[10]	RUNX1	
21	20	M	sRCC	0	46,XY	RUNX1	
22	25	M	sCMML	3	46,XY	RUNX1	
23	4	M	sRAEB	9	na	RUNX1, ASXL1	
24	12	F	sRCC	1	47,XX,del(6)(q25),+del(6)(q25)	ASXL1	
25	16	F	sMDR-AML	40	43-46,XX,dic(5;22)(q11;p11),+6,+8,add(13)(q33),-16, dic(17;21)(p11;p11),ins(17 ;?)(q11 ;?),add(21(p13),+idic(21)(p11)[cp20]	DNMT3A	

F= female, M= male, na= not available
(s)RCC= (secondary) refratory cytopenia of childhood, (s)RAEB= (secondary) refratory anemia with excess of blasts, (s) RAEB-t= (secondary) refractory anemia with excess of blasts in transformation, sMDR-AML= secondary MDS related acute myeloid leukemia
sCMML= secondary chronic myelomonocytic leukemia

NRAS and a *RUNX1* aberration in one patient and two type II aberrations in the other, i.e a *RUNX1* and an *ASXL1* mutation.

Type I/II aberations and cytogenetics

Table 1 and 2 depict an overview of the identified type I and II aberrations in the various cytogenetic subgroups.

No correlation was found between the presence of mutations and the cytogenetic aberrations. Among the 35 MDS patients with monosomy 7, various molecular aberrations were found in 8 cases (Table 3). In 3/13 patients with trisomy 8, a mutation was found. Furthermore, mutations were found in 7/29 of the patients with other karyotypes and in 6/23 of the normal karyotype patients. RUNX1 and CEBPA mutations (all single mutants) were the most frequent mutations, but they did not correlate with a specific karyotype. None of the patients with a complex karyotype (4 primary MDS and 11 secondary MDS) carried a molecular aberration.

As the frequency of mutations was small, no outcome analyses to evaluate the prognostic value of the identified molecular aberrations were made.

DISCUSSION

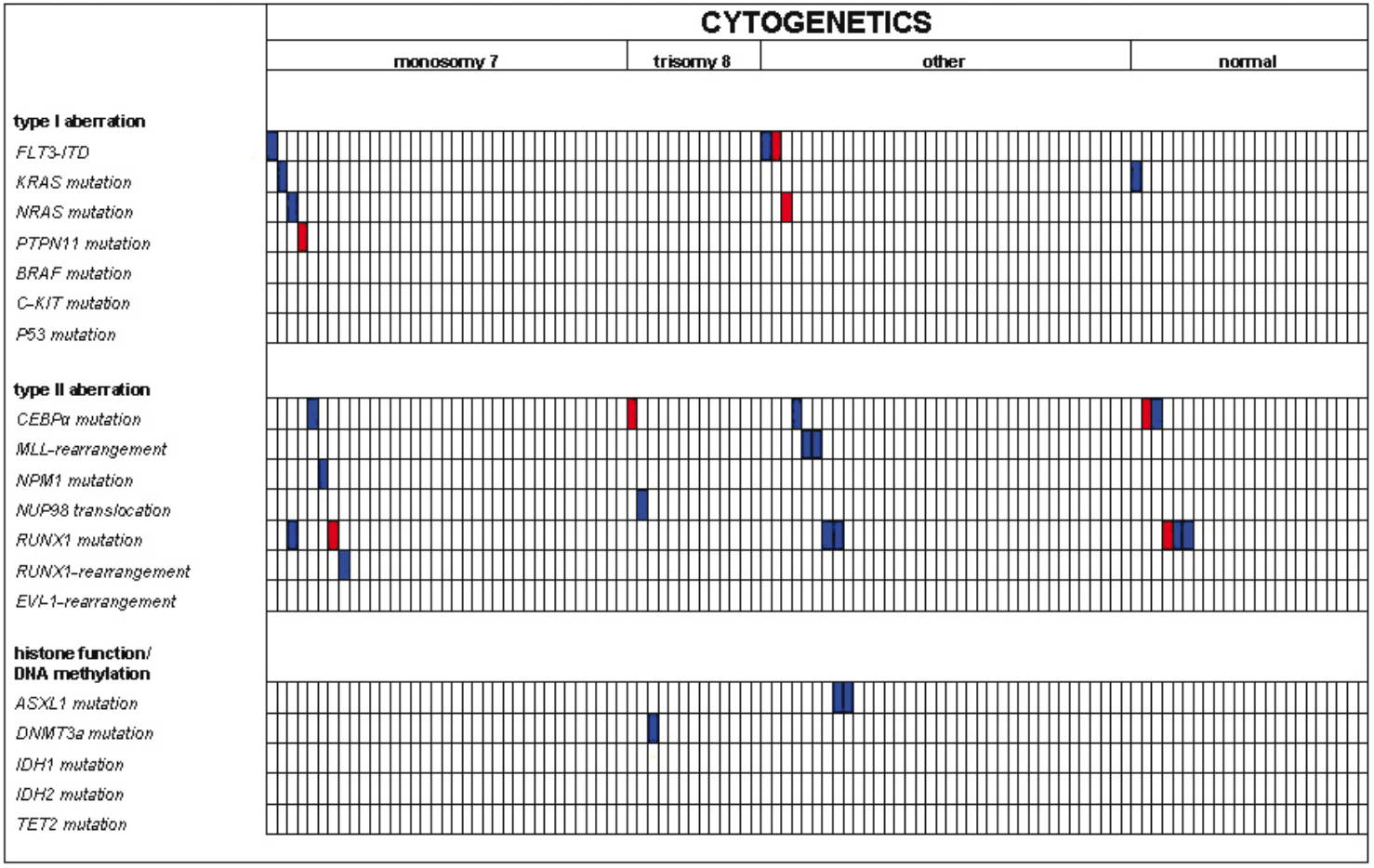
This is the first study of an extensive cohort of childhood MDS cases that aimed to detect molecular aberrations that are involved in the pathogenesis of adult MDS (Table 4) (5-9, 17, 19, 21, 27, 50-51). Our study indicates that in children the occurrence of mutations in primary and secondary MDS seems to be less frequent as in adults, which is most obviously reflected by the frequency of mutations in genes regulating the histone function and DNA methylation. The latter were recently shown to play an important role in adult MDS (Table 4). This emphasizes that, overall, the biology of adult and childhood MDS is different. This is further illustrated by the fact that recently a much lower frequency of mutations in the RNAsplicing machinery in childhood MDS was found as compared to in adults (29-30, 52).

In children with primary MDS we observed type I aberration and type II aberration in about 9% of the cases. In adult studies the frequency of type I aberrations varies between 6 and 22% (6, 9, 53) and of type II aberrations between 5 and 16% (6, 9), and we have to stress that most adult MDS studies on type II aberrations even included only a selection of molecular aberrations and not the broad spectrum we have screened (6, 9).

In our patients with secondary MDS the frequency of type I and II aberrations was 6% and 24%, respectively. The occurrence of type I aberrations in our cohort, which is lower as compared to the largest study so far in adult MDS (type I aberration in 33/140 (24%), type II aberration in 47/140 (34%)), suggests that in childhood MDS especially the proliferation advantage caused by type I aberrations is less prominent as compared to in adults (5).

Pedersen-Bjergaard *et al* described a high frequency of *P53* mutations (24%) in patients with secondary MDS, which were mainly associated with aberrations of chromosome 5 (5). We did not find any *P53* mutations, which may be due to the low frequency of chromosome 5 aberrations in in our representative childhood MDS cohort. In our study, collaborating mutations were found in only 2% of the cases, whereas 13% of the adult secondary MDS cases carried both a type I and type II aberration and 1/140 carried two type II aberrations (5).

Table 3: Cytogenetics and molecular aberrations found in 107 childhood MDS patients



In our cohort 73% of the primary MDS and 83% of the secondary MDS patients had an abnormal karyotype. This further illustrates that, recurrent cytogenetic aberrations may be the major oncogenic event rather that the collaboration between type I and II aberrations. In patients with a normal karyotype, 11/12 primary MDS and 10/11 secondary MDS did not show any molecular aberration which indicates that other unknown responsible mechanisms need to be unraveled in childhood MDS.

We found a low frequency of aberrations in the *MLL, NUP98, TET2, IDH1, IDH2, ASXL1* and *DNMT3A* genes. Recently, these genes have shown to be key-players in the regulation of DNA methylation in several myeloid malignancies and MDS [54-58]. Although epigenetic mutations are not the only factors regulating the DNA methylation, the absence of mutations in these genes in childhood MDS may suggest that aberrant DNA methylation is less important in childhood MDS as compared to adult MDS. This may be due to the fact that epigenetic regulation is related to aging of the hematopoietic stem cell, and subsequent malignant transformation, as illustrated by the observation that MDS increases exponentially with age [57].

This is the first study that systematically investigated a substantial cohort of pediatric MDS samples by international collaboration. The results show that type I and II molecular aberrations, that have been described in pediatric AML and adult MDS/AML, infrequently occur in pediatric MDS. In particular, mutations in genes, playing a role in epigenetic regulation occur in a significantly lower frequency in pediatric MDS as compared to adult MDS. This suggests that other mechanisms drive leukemogenesis in many cases of pediatric MDS compared with the mechanisms in adults. Further research by high throughput analysis, e.g. whole genome sequencing, may further help to unravel the drivers of the biology of childhood MDS [59].

Table 4: Data of this study compared to data of the literature.

	Primary MDS CHildhood (this study)	Primary MDS adults	Secondary MDS Childhood (this study)	Secondary MDS adults	Literature
FLT3	4.5%	0-7%	1.6%	0-3%	Machado-Neto (n=51 pMDS), Bains (n=875 pMDs, n=150 sMDS), Rocqain (n=59 pMDS, n=6 sMDS), Dicker (n=182 pMDS), Bacher (n=338 pMDS), Gritsaev (n=44 pMDS), Pappa (n=64 pMDS), Pedersen (n=140 sMDS)
NRAS	2.3%	6%	1.6%	6%	Dicker (n=176 pMDS), Bacher (n=272 pMDS), Pedersen (n=140 sMDS)
KRAS	0	2%	3.2%	0-5%	Rocquain, (n=59 pMDS, n=6 sMDS), Haferlach, (n=17 pMDS), Pedersen (n=140 sMDS)
PTPN11	2.3%	1%	0	3%	Loh (n=234 pMDS), Pedersen (n=140, sMDS)
c-KIT	0	1-6%	0	1%	Bacher (n=290 pMDS), Lorenzo (n=34 pMDS), Pedersen (n=140 sMDS)
BRAF	0	na	0	2%	Pedersen (n=140 sMDS)
NPM1	0	0-3%	1.6%	0-3%	Bains (n=107 pMDS, n=32 sMDS), Rocquain (n=59 pMDS, n=6 sMDS),Dicker(n=66 pMDS), Andersen (n=89 sMDS)
CEBPA	4.5%	4-6%	4.7%	7% (rearrangement)	Fuchs (n=143 pMDS), Shih (n=35 pMDS), Pedersen (n=140)
MLL-rearrangement	0	0-4%	3.2%	8%	Dicker(n=188 pMDS), Pappa (n=62 pMDS), Pedersen (n=140 sMDS)
RUNX1 rearrangement	0	8 %	1.6%	0	Rocquain (n=59 pMDS, n=6 sMDS)
RUNX1 mutations	4.5%	7-14%	7.9%	16-42%	Dicker (n=188 pMDS), Chen (n=132 pMDS), Rocquain (n=59 pMDS, n=6 sMDS)
NUP98 translocation	0	Case reports	1.6%		
NUP98-NSD1 translocation	0		0		
EVI1-rearrangement	0		0	3%	Pedersen (n=140 sMDS)
P53	0	18% (5q-)	0	24%	Jadersten (n=55 pMDS), Pedersen (n=140 sMDS)
TET2	0	19-26%	0	0	Langemeyer (n=102 pMDS), Rocquain (n=59 pMDS, n=6 sMDS), Delhommeau, (n= 81 pMDS), Kosmider (n=96 pMDS)
ASXL1	0	11-21%	3.2%	50%	Rocquain (n=59 pMDS, n=6 sMDS),Thol (n=193 pMDS), Boultwood (n=144 pMDS), Gelsi-Boyer (n=35 pMDS)
DNMT3A	0	0-8%	1.6%	0	Walter (n=150 pMDS), Lin (n=51 pMDS), Shiba (n=24 pMDS, n=20 sMDS), Thol (n=193 pMDS)
IDH1	0	0-3%	0	16%	Rocquain (n=59 pMDS, n=6 sMDS), Patnaik(n=277 pMDS), Zhou (n=62 pMDS)
IDH2	0	0-9%	0	0	Rocquain (n=59 pMDS, n=6 sMDS), Patnaik(n=277 pMDS), Zhou (n=47 pMDS)

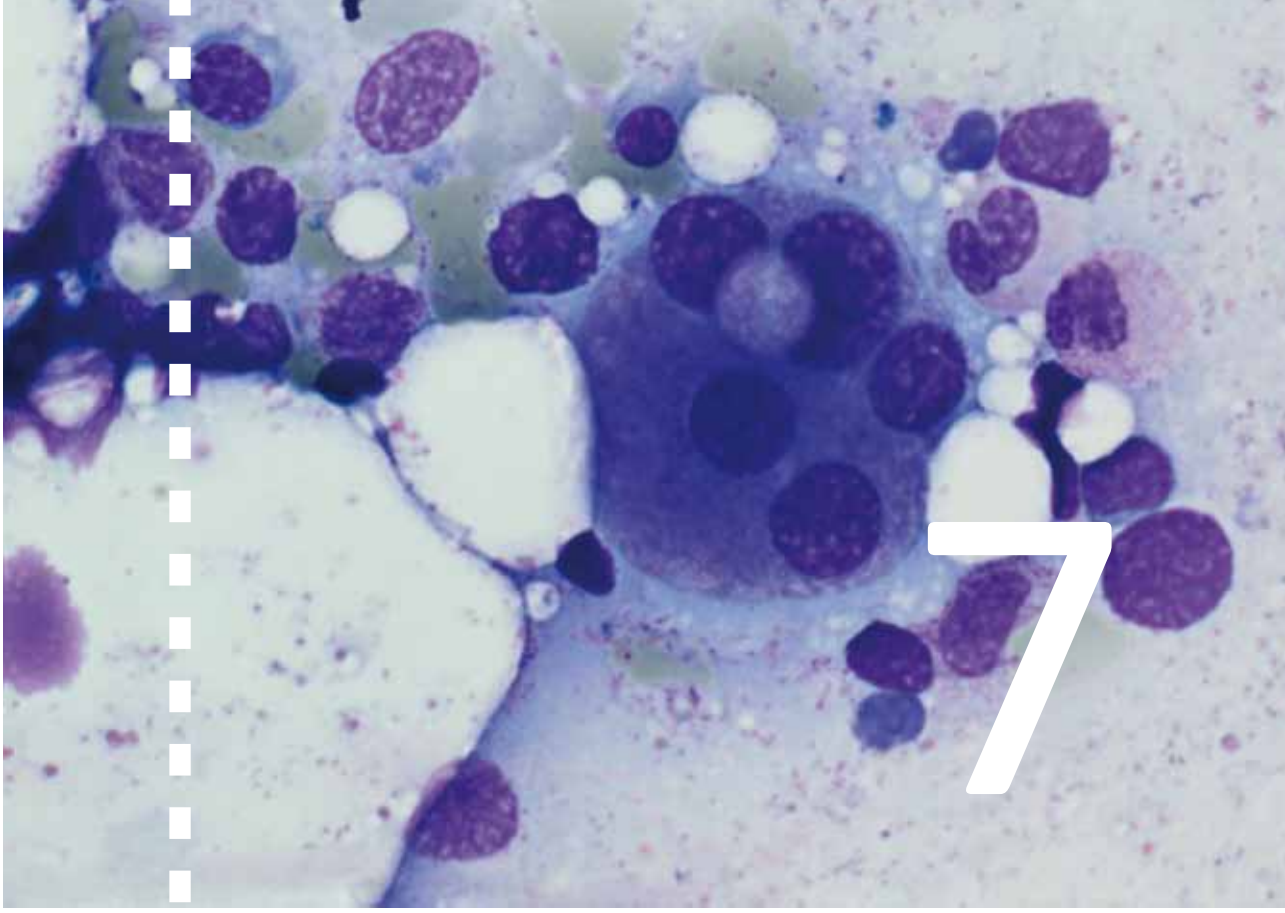
REFERENCES

1. Saitoh K, Miura I, Takahashi N, Miura AB. Fluorescence in situ hybridization of progenitor cells obtained by fluorescence-activated cell sorting for the detection of cells affected by chromosome abnormality trisomy 8 in patients with myelodysplastic syndromes. Blood. 1998 Oct 15;92(8):2886-92.
2. Hasle H, Baumann I, Bergstrasser E, Fenu S, Fischer A, Kardos G, et al. The International Prognostic Scoring System (IPSS) for childhood myelodysplastic syndrome (MDS) and juvenile myelomonocytic leukemia (JMML). Leukemia. 2004 Dec;18(12):2008-14.
3. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. World Health Organization classification of tumours. Pathology and genetics of tumours of haematopoietic and lymphoid tissues: IARC Press, Lyon; 2008.
4. Platzbecker U, Meredyth-Stewart M, Ehninger G. The pathogenesis of myelodysplastic syndromes. Cancer Treatment Reviews. 2007:doi:10.1016/j.crtv.2007.07.021.
5. Pedersen-Bjergaard J, Christiansen DH, Desta F, Andersen MK. Alternative genetic pathways and cooperating genetic abnormalities in the pathogenesis of therapy-related myelodysplasia and acute myeloid leukemia. Leukemia. 2006 Nov;20(11):1943-9.
6. Rocquain J, Carbuccia N, Trouplin V, Raynaud S, Murati A, Nezri M, et al. Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. BMC Cancer. 2010;10:401.
7. Bacher U, Haferlach T, Kern W, Haferlach C, Schnittger S. A comparative study of molecular mutations in 381 patients with myelodysplastic syndrome and in 4130 patients with acute myeloid leukemia. Haematologica. 2007 Jun;92(6):744-52.
8. Bains A, Luthra R, Medeiros LJ, Zuo Z. FLT3 and NPM1 mutations in myelodysplastic syndromes: Frequency and potential value for predicting progression to acute myeloid leukemia. Am J Clin Pathol. 2011 Jan;135(1):62-9.
9. Dicker F, Haferlach C, Sundermann J, Wendland N, Weiss T, Kern W, et al. Mutation analysis for RUNX1, MLL-PTD, FLT3-ITD, NPM1 and NRAS in 269 patients with MDS or secondary AML. Leukemia. 2010 Aug;24(8):1528-32.
10. Fuchs O, Provaznikova D, Kocova M, Kostecka A, Cvekova P, Neuwirtova R, et al. CEBPA polymorphisms and mutations in patients with acute myeloid leukemia, myelodysplastic syndrome, multiple myeloma and non-Hodgkin's lymphoma. Blood cells, molecules & diseases. 2008 May-Jun;40(3):401-5.
11. Haferlach C, Bacher U, Haferlach T, Dicker F, Alpermann T, Kern W, et al. The inv(3)(q21q26)/t(3;3)(q21;q26) is frequently accompanied by alterations of the RUNX1, KRAS and NRAS and NF1 genes and mediates adverse prognosis both in MDS and in AML: a study in 39 cases of MDS or AML. Leukemia. 2011 May;25(5):874-7.
12. Loh ML, Vattikuti S, Schubbert S, Reynolds MG, Carlson E, Lieuw KH, et al. Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis. Blood. 2004 Mar 15;103(6):2325-31.
13. Lorenzo F, Nishii K, Monma F, Kuwagata S, Usui E, Shiku H. Mutational analysis of the KIT gene in myelodysplastic syndrome (MDS) and MDS-derived leukemia. Leukemia research. 2006 Oct;30(10):1235-9.
14. Pappa V, Young BD, Economopoulos T, Papageorgiou E, Panani A, Lilington D, et al. Absence of MLL gene rearrangement in de novo myelodysplastic syndromes (MDS). Annals of hematology. 2004 Mar;83(3):170-5.
15. Boultwood J, Perry J, Pellagatti A, Fernandez-Mercado M, Fernandez-Santamaria C, Calasanz MJ, et al. Frequent mutation of the polycomb-associated gene ASXL1 in the myelodysplastic syndromes and in acute myeloid leukemia. Leukemia. 2010 May;24(5):1062-5.
16. Chen CY, Lin LI, Tang JL, Ko BS, Tsay W, Chou WC, et al. RUNX1 gene mutation in primary myelodysplastic syndrome--the mutation can be detected early at diagnosis or acquired during disease progression and is associated with poor outcome. Br J Haematol. 2007 Nov;139(3):405-14.
17. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, et al. Mutation in TET2 in myeloid cancers. The New England journal of medicine. 2009 May 28;360(22):2289-301.
18. Gelsi-Boyer V, Trouplin V, Adelaide J, Bonansea J, Cervera N, Carbuccia N, et al. Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. Br J Haematol. 2009 Jun;145(6):788-800.
19. Jadersten M, Saft L, Pellagatti A, Gohring G, Wainscoat JS, Boultwood J, et al. Clonal heterogeneity in the 5q- syndrome: p53 expressing progenitors prevail during lenalidomide treatment and expand at disease progression. Haematologica. 2009 Dec;94(12):1762-6.
20. Kosmider O, Gelsi-Boyer V, Cheok M, Grabar S, Della-Valle V, Picard F, et al. TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs). Blood. 2009 Oct 8;114(15):3285-91.
21. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet. 2009 Jul;41(7):838-42.
22. Lin J, Yao DM, Qian J, Chen Q, Qian W, Li Y, et al. Recurrent DNMT3A R882 mutations in Chinese patients with acute myeloid leukemia and myelodysplastic syndrome. PLoS One. 2011;6(10):e26906.

23. Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tonnissen ER, et al. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. Nat Genet. 2010 Aug;42(8):665-7.
24. Patnaik MM, Hanson CA, Hodnefield JM, Lasho TL, Finke CM, Knudson RA, et al. Differential prognostic effect of IDH1 versus IDH2 mutations in myelodysplastic syndromes: a Mayo Clinic study of 277 patients. Leukemia. 2012 Jan;26(1):101-5.
25. Thol F, Friesen I, Damm F, Yun H, Weissinger EM, Krauter J, et al. Prognostic significance of ASXL1 mutations in patients with myelodysplastic syndromes. J Clin Oncol. 2011 Jun 20;29(18):2499-506.
26. Thol F, Winschel C, Ludeking A, Yun H, Friesen I, Damm F, et al. Rare occurrence of DNMT3A mutations in myelodysplastic syndromes. Haematologica. 2011 Dec;96(12):1870-3.
27. Walter MJ, Ding L, Shen D, Shao J, Grillot M, McLellan M, et al. Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. Leukemia. 2011 Jul;25(7):1153-8.
28. Zou Y, Zeng Y, Zhang DF, Zou SH, Cheng YF, Yao YG. IDH1 and IDH2 mutations are frequent in Chinese patients with acute myeloid leukemia but rare in other types of hematological disorders. Biochem Biophys Res Commun. 2010 Nov 12;402(2):378-83.
29. Thol F, Kade S, Schlarmann C, Loffeld P, Morgan M, Krauter J, et al. Frequency and prognostic impact of mutations in SRSF2, U2AF1, and ZRSR2 in patients with myelodysplastic syndromes. Blood. 2012 Apr 12;119(15):3578-84.
30. Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. Nature. 2011 Oct 6;478(7367):64-9.
31. Paulsson K, Heidenblad M, Strombeck B, Staaf J, Jonsson G, Borg A, et al. High-resolution genome-wide array-based comparative genome hybridization reveals cryptic chromosome changes in AML and MDS cases with trisomy 8 as the sole cytogenetic aberration. Leukemia. 2006 May;20(5):840-6.
32. Hasle H, Niemeyer CM, Chessells JM, Baumann I, Bennett JM, Kerndrup G, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia. 2003 Feb;17(2):277-82.
33. Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. World Health Organization classification of tumours. Pathology and genetics. Tumours of Haematopoietic and Lymphoid tissues: IARC Press, Lyon; 2001.
34. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003 Sep 1;21(17):3262-8.
35. Van Vlierberghe P, van Grotel M, Tchinda J, Lee C, Beverloo HB, van der Spek PJ, et al. The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia. Blood. 2008 May 1;111(9):4668-80.
36. EWOG-MDS. Clinical Trial Protocol EWOG-MDS 2006. Prospective non-randomized multi-center study for epidemiology and characterization of Myelodysplastic Syndromes (MDS) and Juvenile Myelomonocytic Leukemia (JMML) in childhood. 2007.
37. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood. 1998 Oct 1;92(7):2322-33.
38. Gohring G, Michalova K, Beverloo HB, Betts D, Harbott J, Haas OA, et al. Complex karyotype newly defined: the strongest prognostic factor in advanced childhood myelodysplastic syndrome. Blood. 2010 Nov 11;116(19):3766-9.
39. Balgobind BV, Van Vlierberghe P, van den Ouweland AM, Beverloo HB, Terlouw-Kromosoeto JN, van Wering ER, et al. Leukemia-associated NF1 inactivation in patients with pediatric T-ALL and AML lacking evidence for neurofibromatosis. Blood. 2008 Apr 15;111(8):4322-8.
40. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, Meijer J, van Oosterhoud S, van Putten WL, Valk PJ, et al. Biallelic mutations in the CEBPA gene and low CEBPA expression levels as prognostic markers in intermediate-risk AML. Hematol J. 2003;4(1):31-40.
41. Caligiuri MA, Strout MP, Schichman SA, Mrozek K, Arthur DC, Herzig GP, et al. Partial tandem duplication of ALL1 as a recurrent molecular defect in acute myeloid leukemia with trisomy 11. Cancer Res. 1996 Mar 15;56(6):1418-25.
42. Hollink IH, van den Heuvel-Eibrink MM, Zimmermann M, Balgobind BV, Arentsen-Peters ST, Alders M, et al. Clinical relevance of Wilms tumor 1 gene mutations in childhood acute myeloid leukemia. Blood. 2009 Jun 4;113(23):5951-60.
43. Hollink IH, Zwaan CM, Zimmermann M, Arentsen-Peters TC, Pieters R, Cloos J, et al. Favorable prognostic impact of NPM1 gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML. Leukemia. 2009 Feb;23(2):262-70.
44. Kiyoi H, Naoe T, Yokota S, Nakao M, Minami S, Kuriyama K, et al. Internal tandem duplication of FLT3 associated with leukocytosis in acute promyelocytic leukemia. Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). Leukemia. 1997 Sep;11(9):1447-52.
45. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001 Apr 15;97(8):2434-9.
46. Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, Zimmermann M, Peeters JK, Valk PJ, et al. Characterization of CEBPA mutations and promoter

hypermethylation in pediatric acute myeloid leukemia. Haematologica. 2011 Mar;96(3):384-92.

47. Balgobind BV, Hollink IH, Arentsen-Peters ST, Zimmermann M, Harbott J, Beverloo HB, et al. Integrative analysis of type-I and type-II aberrations underscores the genetic heterogeneity of pediatric acute myeloid leukemia. Haematologica. 2011 Oct;96(10):1478-87.
48. 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.
49. Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, Pratcorona M, Abbas S, Kuipers JE, et al. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. Blood. 2011 Sep 29;118(13):3645-56.
50. Andersen MT, Andersen MK, Christiansen DH, Pedersen-Bjergaard J. NPM1 mutations in therapy-related acute myeloid leukemia with uncharacteristic features. Leukemia. 2008 May;22(5):951-5.
51. Pardanani A, Patnaik MM, Lasho TL, Mai M, Knudson RA, Finke C, et al. Recurrent IDH mutations in high-risk myelodysplastic syndrome or acute myeloid leukemia with isolated del(5q). Leukemia. 2010 Jul;24(7):1370-2.
52. Hirabayashi S, Flotho C, Moetter J, Heuser M, Hasle H, Gruhn B, et al. Spliceosomal gene aberrations are rare, coexist with oncogenic mutations, and are unlikely to exert a driver effect in childhood MDS and JMML. Blood. 2012 Mar 15;119(11):e96-9.
53. Bacher U, Haferlach C, Kern W, Haferlach T, Schnittger S. Prognostic relevance of FLT3-TKD mutations in AML: the combination matters--an analysis of 3082 patients. Blood. 2008 Mar 1;111(5):2527-37.
54. Bernt KM, Armstrong SA. A role for DOT1L in MLL-rearranged leukemias. Epigenomics. 2011 Dec;3(6):667-70.
55. Fathi AT, Abdel-Wahab O. Mutations in epigenetic modifiers in myeloid malignancies and the prospect of novel epigenetic-targeted therapy. Adv Hematol. 2012;2012:469592.
56. Fisher CL, Pineault N, Brookes C, Helgason CD, Ohta H, Bodner C, et al. Loss-of-function Additional sex combs like 1 mutations disrupt hematopoiesis but do not cause severe myelodysplasia or leukemia. Blood. 2010 Jan 7;115(1):38-46.
57. Klauke K, de Haan G. Polycomb group proteins in hematopoietic stem cell aging and malignancies. International journal of hematology. 2011 Jul;94(1):11-23.
58. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell. 2011 Jan 18;19(1):17-30.
59. Damm F, Bunke T, Thol F, Markus B, Wagner K, Gohring G, et al. Prognostic implications and molecular associations of NADH dehydrogenase subunit 4 (ND4) mutations in acute myeloid leukemia. Leukemia. 2011.



7

IER3 EXPRESSION IN CHILDHOOD MDS

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Submitted

Illustration: MDS, dysmegakaryopoiesis (BM)

ABSTRACT

Background: Childhood myelodysplastic syndrome (MDS) is a rare disease accounting for less than 5% of all hematological malignancies. In about 50% of the MDS cases an abnormal karyotype is found by conventional karyotyping, of which chromosome 6 is involved in 10%. The immediate-early-response 3 (*IER3)* gene, which is located on chromosome 6p21, encodes for a glycoprotein that plays a role in the regulation of apoptosis and cell cycle progression. Recently, it was shown that *IER3* gene aberrations frequently occur in adult MDS patients, which are not restricted to patients with chromosome 6 aberrations and that low *IER3* expression was associated with a worse outcome. Therefore, we investigated the frequency and prognostic impact of *IER3* expression in childhood MDS.

Methods: *IER3* mRNA expression was determined by quantitative real-time PCR in 58 childhood MDS patients of which 17 carried a chromosome 6 aberration, and in normal bone marrow (n=8). In addition, methylation-specific-PCR (MSP) was performed to investigate the methylation status of the promoter region of the *IER3* gene, as a plausible cause for the downregulation of *IER3*.

Results: Median *IER3* mRNA expression was 0.9% in MDS (range 0.01-73.3% relative to *GAPDH* expression) and 3.3% in normal bone marrow (range 0.81-85.5% relative to *GAPDH* expression) (p=0.05). A more than 4-fold decrease in *IER3* expression below the mean of healthy controls was found in 74% (43/58) of the childhood MDS patients. There was no difference in *IER3* mRNA expression between MDS patients with or without chromosome 6 aberrations (MWU p= 0.89). Also, no difference in *IER3* mRNA expression was found between the different WHO-subtypes or between primary versus secondary MDS. Three patients with a chromosome 6p21 rearrangement in the *IER3* region based on genomic profiling (array-CGH) showed expression-levels in the range of normal bone marrow. Low *IER3* mRNA expression was associated with adverse outcome in childhood MDS patients (Cox regression analysis estimated hazard ratio 0.73, p=0.027, 95% CI-interval 0.55-0.97). The low *IER3* expression appeared not to be due to hypermethylation of its promoter region.

Conclusion: *IER3* expression is low in childhood MDS patients with and without chromosome 6 aberrations and this low expression is associated with poor outcome. However this seems to be due to therapy related mortality, rather than by the increased risk of relapse. The *IER3* downregulation is not regulated by hypermethylation of the *IER3* promoter region.

INTRODUCTION

In childhood, myelodysplastic syndrome (MDS) is a rare disease accounting for less than 5% of all hematological malignancies (1). The most common cytogenetic abnormalities in childhood MDS are monosomy 7 and trisomy 8 (2-3). Recently, chromosome 6 aberrations were identified as a recurrent genetic abnormality in childhood MDS cases with an aberrant karyotype (2, 4). It is unknown which specific molecular aberrations associated to chromosome 6 abnormalities are relevant for the development and outcome of childhood MDS.

Genome-wide approaches proved to be powerful tools to dissect haematological malignancies molecularly. Recently, through array-CGH, Steensma *et al* identified a breakpoint in the *IER3* gene (immediate early response 3 gene, previously named *IEX-1*) in an adult MDS patient with t(6;9)(p21.3;q34). Subsequent analysis of 204 adult MDS cases showed eight additional patients with *IER3* rearrangements. Aberrant expression of the *IER3* gene, localized at chromosome 6p21.33, was found in 61% of the adult MDS patients, even in cases without a 6p rearrangement (5). In addition, it was shown that low *IER3* expression was associated with a worse outcome in adult MDS (6). So far, no information on the relevance of *IER3* expression in pediatric MDS is available.

Therefore, we performed a comprehensive study of 58 pediatric MDS cases collected by the European Working Group of MDS and JMML in Childhood (EWOG-MDS), including 17 cases with chromosome 6 abnormalities by conventional karyotyping (among which 3 cases with rearrangements in the *IER3* region determined by array-CGH) to study the role of *IER3* expression in pediatric MDS.

PATIENTS

In July 2009 in the database of the EWOG-MDS 530 patients with MDS are registered, of which ~45% have an abnormal karyotype, including 30 which carried a chromosome 6 aberration. In 23/30 of these cases material was available for array-CGH. Of these 23 patients, 13 had primary MDS (refractory cytopenia of childhood (RCC) n=1, refractory anemia with excess of blasts (RAEB) n=6, refractory cytopenia with excess of blasts in transformation (RAEB-t) n=6) and 10 patients had secondary MDS (RCC n= 5, RAEB n=3, RAEB-t n=2). Seven secondary MDS cases occurred after previous cancer treatment and three in patients with constitutional bone marrow failure syndromes.

IER3 mRNA expression levels were measured in 58 pediatric MDS patients, selected on the availability of material. This cohort included 17 of the 23 aforementioned patients with chromosome 6 abnormalities and 41 additional patients without chromosome 6 aberration based upon conventional karyotyping (Table 1). Of these 58 patients, 8 patients were not transplanted: i.e. 3 patients with familial MDS who had stable disease, 3 patients who died due to disease progression before they could be transplanted, one patient died due to infection before the transplantation and one patient was successfully treated with immunosuppressive therapy. As control material, the normal bone marrow of eight children in the same age range was used.

Table 1: Patients characteristics of the MDS patients with and without chromosome 6 aberration based on conventional karyotyping included for IER3 expression analysis.

	Patients with chromosome 6 aberration N=17	Patients without chromosome 6 aberration N=41
Gender (male/female)	12/5	21/20
Age at diagnosis (years)	1.8-16.5	0.8-17.5
Median age	6.0	
WHO		
Primary		
- RCC	2	10
- RAEB	3	19
- RAEB-t	4	0
Secondary		
- RCC	2	7
- RAEB	4	4
- RAEB-t	1	1
Number of patients transplanted	15	37
IER3 expression relative to GAPDH (%)	0.1-20.2	0.02-73.2

RCC = refractory cytopenia of childhood
RAEB = refractory anemia with excess of blasts
RAEB-t = refractory cytopenia with excess of blasts in transformation

METHODS

Array-CGH

Genomic profiling was performed, using the Agilent (Agilent Technologies, Palo Alto, California, USA) 105k Array-CGH platform, on DNA from 23 childhood MDS patients with chromosome 6 abnormalities, as previously described (9-10). Briefly, patient genomic DNA (2 mg) and reference male human genomic DNA (2 mg) (Promega) were labelled with Cy5 and Cy3 dyes according to standard random priming protocols (Bioprime Labeling Kit, Invitrogen, Carlsbad, CA, USA). The DNAs were combined, denatured, and applied to the Agilent Array, according to the manufacturer's protocol. DNA hybridization and washes were performed as recommended, and the slides were scanned on a GenePix 4000B Microarray Scanner (Axon Instruments, Union City, CA, USA). Arrays were analyzed using feature extraction software (version 8.1, Agilent). The data were imported in array-CGH analytics software of Agilent (11).

Quantitative real-time PCR

cDNA was prepared as previously described (7). Quantitative real-time PCR (RQ-PCR) was performed on a total of 58 pediatric MDS patients with available cDNA. An ABI PRISM 7900HT thermal cycler (Applied Biosystems, Foster City, CA, USA) was used. Primers for *IER3* are described in Table 2. For expression analysis of *IER3* SYBRgreen was used. The

IER3 expression was compared to *GAPDH*, with primers as previously described (7). The average cycle threshold (Ct) value was used to calculate mRNA expression levels of *IER3* relative to the expression level of the reference gene (*GAPDH*) using the comparative Cycle time (ΔCt) method (8).

Methylation Specific PCR

To investigate the methylation status of *IER3*, methylation specific PCR (MS-PCR) was performed, as previously described (12) The primers were located in the CpG-island of the promoter sequence (designated M/U) (Table 2). Unmodified genomic DNA was used to test the specificity of the primers for bisulfite converted DNA. One DNA sample was first treated with DNA methylase SSS1 and methyl donor SAM (M0226S New England Biolabs, Ipswich, MA, USA) and then bisulfite converted, creating a hypermethylated sample as a positive control for methylation. Bisulfite converted unmethylated DNA of a healthy, adult male donor was used as negative control. The specificity of the methylation (M) and unmethylation (U) specific primers was tested on a dilution range with a mix of M and U DNA. For this analysis, eighteen MDS samples were selected: nine samples with the highest *IER3* mRNA expression and nine samples with the lowest *IER3* mRNA expression as measured by RQ-PCR.

Table 2: Primer sequences of IER3 and GAPDH used for RQ-PCR and Methylation Specific PCR.

Primer	Sequence (5'-3')
IER3 forward	GCC GCA GGG TTC TCT A
IER3 reverse	GGG CTC CGA AGT CAG A
MS-PCR region 1 *IER3* methylated forward	CGT CGG AAT TTT TAG TTC
MS-PCR region 1 *IER3* methylated reverse	AAA TAA AAA TCG ACT ACC G
MS-PCR region 1 *IER3* unmethylated forward	TGT TGG AAT TTT TAG TTT
MS-PCR region 1 *IER3* unmethylated reverse	AAA TAA AAA TCA ACT ACC A
GAPDH forward	GTC GGA GTC AAC GGA TT
GAPDH reverse	AAG CTT CCC GTT CTC AG

Statistical analyses:

The Mann-Whitney-U test (MWU) was used for the comparison of the *IER3* expression in two different groups and the Kruskal-Wallis-test was used for the comparison of more than two groups. Low *IER3* expression was defined as a more than 4-fold decrease in expression compared with the mean *IER3* expression of healthy controls, as previously described (5). Probabilities of overall survival were estimated by the method of Kaplan Meier and compared by the use of log-rank test. Cox regression analysis was performed to investigate the influence of *IER3* expression on overall survival. Cumulative incidence of relapses (CIR) were constructed by the method of Kalbfleisch and Prentice (13). The survival analyses were an intention to treat analyses. All analyses were performed with SPSS Statistics version 17.0. All used tests were two-tailed and a value of less than 0.05 was considered statistically significant.

RESULTS

Array-CGH was performed in 23 patients. In 3/23 a breakpoint in the area where IER3 is located was detected (Table 3) (Figure 1). In patient 1 with a ring chromosome 6 the breakpoints could be identified precisly. Conventional karyotyping of patient 2 identified a trisomy 6, but array-CGH revealed that this was only an amplification of a part of chromosome 6. In the third case the breakpoint at the short arm of the chromosome 6 could be identified more exact by array-CGH. In these three cases the *IER3* gene was located in or near the breakpoint. To investigate whether this gene is involved in a larger extend, we analyzed the *IER3* expression in a larger cohort of childhood MDS.

The median *IER3* mRNA expression in the pediatric MDS patients was 0.92% (range 0.01-73,2% relative to *GAPDH*) as compared to 3.38% in normal bone marrow (0.81-85.5% relative to *GAPDH* (Mann-Whitney U test: p-value: 0.05) (Figure 2A). A low *IER3* expression (a more than 4 fold decrease in expression compared with the mean *IER3* expression of healthy controls) was found in 74% (43/58) of the childhood MDS patients.

No difference in *IER3* mRNA expression was found comparing the patients with (n=17) or without a karyotypic chromosome 6 aberration (n=41); median *IER3* expression levels were 0.99% (range 0.01-20.2%) and 0.76% (range 0.02-73.2%) respectively (ns) (Figure 2B). *IER3* mRNA expression of the 3 patients with aberrant chromosome 6, with a rearrangement in the *IER3* gene region determined by array-CGH, was not different from the patients without a rearrangement (ns). Unfortunately, of those specific 3 cases cytospins were not available to perform FISH analysis.

Table 3: Clinical data of the patients with a rearrangement of the *IER3* gene region at 6p21 identified by array-CGH.

	Patient 1	Patient 2	Patient 3
Gender	M	F	F
Age at diagnosis (years)	14	2. 8	4.3
FAB classification	Primary RCC	Primary RAEB	Secondary RCC (after chemotherapy)
Hemoglobin (g/dl)	10.6	10.9	7.5
WBC (x 10^9/l)	3.7	6.4	3.0
Platelets (x 10^9/l)	77	36	18
Blasts BM (%)	1	18	3
Cytogenetics	46,XY, idic(6)(p2??2q?) [17],/47, idem,+idic(6) [4]/45, XY, -6[5]	50,XX,+4,+6,+8,+21	46,XX,der(6)add(6)(p25) ?ins(6;?)(q?15-q?21;?)
Findings array-CGH regarding to chromosome 6	del 6q12-qter, del 6p21-pter, amplification 6p21-6q12	amplification 6p12.3 -6p21	del6p21-pter
Course	SCT Died: TRM FU: 1.6 years	SCT Alive in CR FU: 8 years	SCT Alive in CR FU: 7.7 years

M= male, F= female, RAEB= refractory anemia with excess of blasts, RCC= refractory cytopenia of childhood, SCT= stem cell transplantation, CR= complete remission, FU= follow up

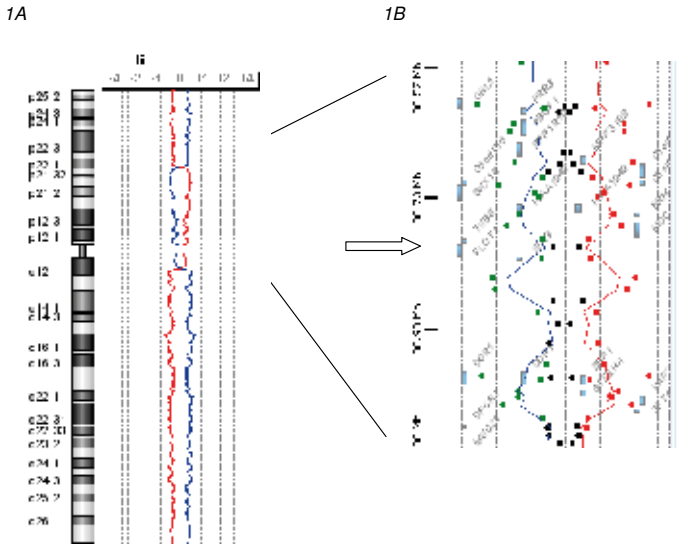


Figure 1: Array-CGH of one patient with rearrangement in *IER3* region. *Figure 1A:* Chromosome 6 ideogram, and corresponding array-CGH plot of the ratio of patient 1 DNA versus control DNA (blue tracing) in a dye swap experiment (red tracing) from an array-CGH experiment (105k oligo-array-CGH platform). The ideogram shows a deletion of 6q12-qter, a deletion of 6p21-pter and an amplification of 6p21-6q12. Arrow indicates localisation of *IER3*, which is located in the region of the breakpoint at 6p21. Figure 1B: A detailed overview of the *IER3* region on chromosome 6 of the same patient.

No significant difference in *IER3* mRNA expression was found between the different WHO-subtypes of MDS (median expression RCC: 1.17%, RAEB: 0.47%, RAEB-t: 0.54% and secondary MDS: 1.9%, Kruskal-Wallis-test: ns) (Figure 2C). *IER3* mRNA expression tended to be higher in secondary MDS (median 1.9%, range 0.01-73.2%) as compared to primary MDS (median 0.69%, range 0.05-12.48%) (MWU: p=0.05).

To evaluate whether low *IER3* mRNA expression was regulated by hypermethylation of the CpG islands of the promotor region of the *IER3* gene, we performed methylation specific PCR (MSP) in a subset of 18 patients with the lowest (n=9) and highest (n=9) *IER3* mRNA expression as determined by RQ-PCR. None of the patients revealed hypermethylation of the *IER3* gene (Figure 3).

Five years overall survival of patients with low *IER3* expression was 47% compared to 85% in the other patients. Univariate Cox regression analysis showed that a decrease in *IER3* expression was associated with a decrease in overall survival (estimated Hazard ratio 0.73, p=0.027, 95% CI interval: 0.55-0.97) (Figure 4). Unfortunately, the numbers of patients were too small to allow multivariate analysis. The worse overall outcome in the patient group with low *IER3* expression was not caused by a higher cumulative incidence of relapse (9/43 of the patients with low *IER3* expression, CIR at 5 year: 0.22, 3/15 of the other patients relapsed, CIR at 5 year: 0.18), but rather by a higher therapy related mortality rate (11/43 patients with low *IER3* expression died due to TRM versus 1/15 of the other patients). Three of the twelve patients who died due to TRM were diagnosed as

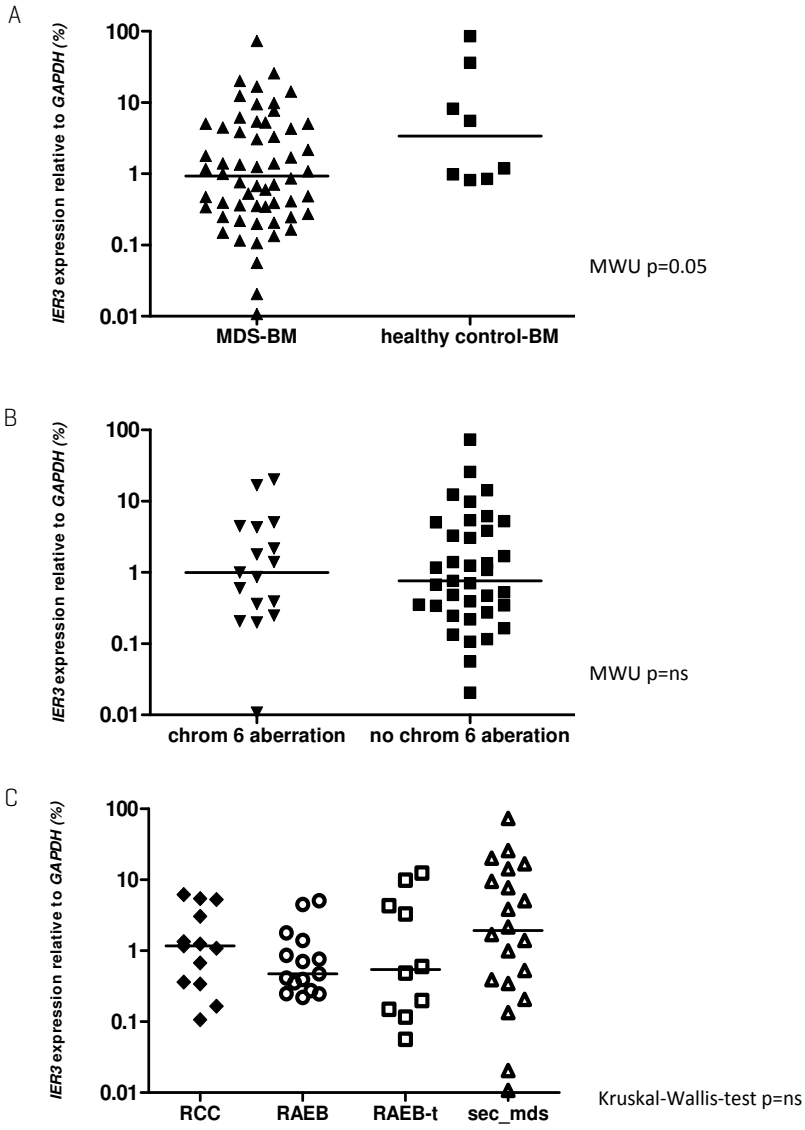


Figure 2: *IER3* expression in childhood MDS. Figure 2A: *IER3* expression in MDS versus normal bone marrow. Expression levels of *IER3* relative to *GAPDH* expression in bone marrow of 58 pediatric MDS patients compared to normal bone marrow of 8 healthy children. The horizontal lines represent the median values. Figure 2B: *IER3* expression in MDS with and without chromosome 6 aberration. Expression levels of *IER3* relative to *GAPDH* expression in bone marrow of 17 pediatric MDS patients with a chromosome 6 aberration compared to bone marrow of 41 pediatric MDS patients without a chromosome 6 aberration. The horizontal lines represent the median values. Figure 2C: *IER3* expression in different FAB types of MDS. Expression levels of *IER3* relative to *GAPDH* expression in bone marrow according to FAB subtype: RCC n= 13, RAEB n= 15, RAEB-t n= 10 and secondary MDS n= 20. The horizontal lines represent the median values.

RCC, 5 as RAEB and 4 were diagnosed with secondary MDS. All, except one patient who died due to infection, died after transplantation.

DISCUSSION

In pediatric MDS prognostic relevant recurrent clonal numerical cytogenetic aberrations, especially monosomy 7 and trisomy 8, occur in 50% of the cases [2-4]. In addition, Göhring *et al* recently described, that the presence of a structurally complex karyotype is the strongest independent predictor for poor outcome in children with advanced MDS [4]. Recently, we identified that chromosome 6 aberrations occur in about 5% of the MDS cases and that they are involved in 10% of all cases with an abnormal karyotype. Until now, neither the prognostic role of chromosome 6 abnormalities nor the role of genes involved in chromosome 6 abnormalities are clarified in childhood MDS.

Recently, in adult MDS, downregulated expression of the *IER3* gene, located on 6p21.3 was described [5]. Others showed that differentially expressed *IER3* is associated with clinically relevant subgroups and outcome in adult MDS [6, 14]. Our present study is the first on the impact of *IER3* mRNA expression in childhood MDS. Our cohort revealed a large inter-patient variety in *IER3* mRNA expression, similar to what has been reported in adult MDS [5]. Concordant with adult MDS studies, we found no difference in expression between the patients with or without a chromosome 6 aberration in children [5].

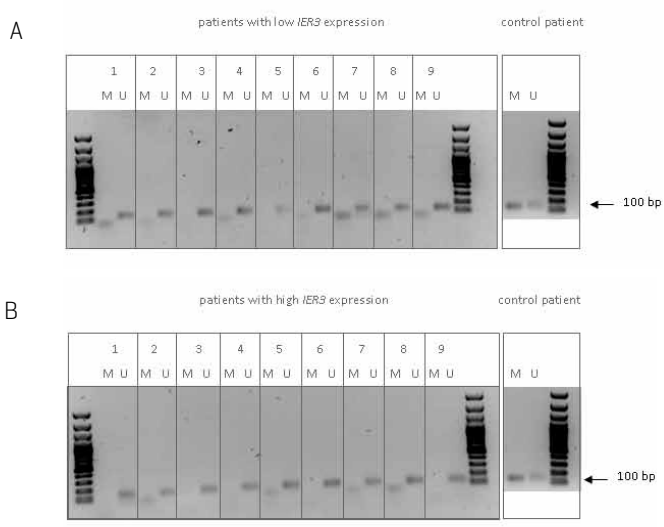


Figure 3: Methylation specific PCR. *Figure 3A: Methylation specific PCR in nine patients with low IER3 expression.* Figure 3B: Methylation specific PCR in nine patients with high IER3 expression. Methylation specific PCR in patients with low IER3 expression (n=9)(Figure 3A) and high IER3 expression (n=9) (Figure 3B). M: methylated, U unmethylated. In patients with low and high IER3 expression no bands of the expected size (100 bp) were observed are seen using the methylated specific primers (the smaller bands are most likely primer dimer bands) and bands are seen using the unmethylated primers. The control patient bands of the same size are found using both the methylated and the unmethylated primers as expected (arrow: expected product size of 100bp)

Furthermore, we found no significant difference in the *IER3* mRNA expression between MDS-RCC and more advanced MDS in children. Limitation of our study is, that due to the low absolute number of childhood MDS cases, even after including most recent European pediatric patients, multivariate analyses are not feasible. Nevertheless, Cox regression analysis in our cohort showed that overall survival is significantly impaired in children with low *IER3* expression similar to what has been found in adult MDS [6] .

In adults the reason for the worse outcome of the patients with low *IER3* expression is not further specified. We show here that the worse outcome of the childhood cases with low *IER3* expression was determined by a higher therapy related mortality rate rather than by a higher relapse rate.

As indicated, in adults it has not been described why downregulation of the *IER3* gene results in a lower probability of survival. The *IER3* gene, formerly known as *IEX-1* or p22/PRG1, is characterized by the fact that expression increases rapidly in response to cellular stress. The gene encodes a 27-kDa glycoprotein regulating death receptor-induced apoptosis. *IER3* expression is regulated by multiple upstream events like aberrant expression of the transcription factors *p53*, *NF-κB* and *c-MYC*, which bind at the promoter region of the *IER3* gene and which play a role in the regulation of apoptosis [15]. IER3 is also a target of ERK and in this way cell growth is regulated [16]. Moreover, IER3 suppresses the production of reactive oxygen species and protects cells from apoptosis [17]. Whether the effect of IER3 on apoptosis is enhancing or suppressive depends on the cellular context and cell type as well as the apoptosis stimulus [15, 18]. It has been suggested that downregulation of *IER3* is the result of mutations in the promoter region at the binding sites of these transcription factors, but Steensma *et al*

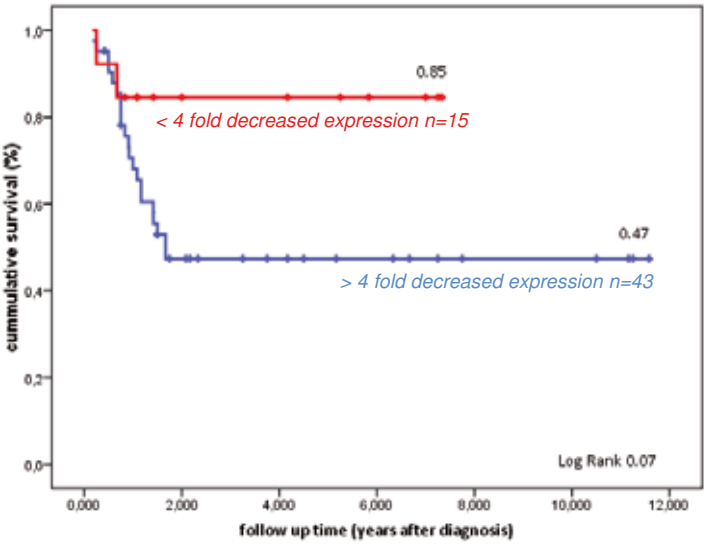


Figure 4. Kaplan Meier plot. Plot showing the overall survival for all MDS patients with low *IER3* expression (n=43) compared to the other patients (n=15) Low IER3 expression is defined as a more than 4-fold decrease in expression compared to the mean of healthy controls (log rank p=0.07)

sequenced the *IER3* coding and promoter regions in adult MDS patients and revealed no point mutations. Therefore, we did not search for *IER3* mutations in our pediatric cohort. As to date, no information is available regarding other mechanisms of downregulation in poor risk MDS patients, we performed *TP53* mutation analysis but found only 1 case in our cohort (data not shown), indicating that this may not be the most prominent regulating event. In addition, we showed that *IER3* downregulation is not regulated by hypermethylation of the promoter region.

We conclude that in the majority of pediatric MDS patients, *IER3* expression is relatively low and expression is not regulated by hypermethylation of the promoter region. Low *IER3* expression is not associated with structural chromosome 6 aberrations and is associated with adverse outcome, due to a high number of treatment related deaths. To understand the mechanism and ultimate pathobiological effect of *IER3* downregulation in pediatric MDS further studies are required.

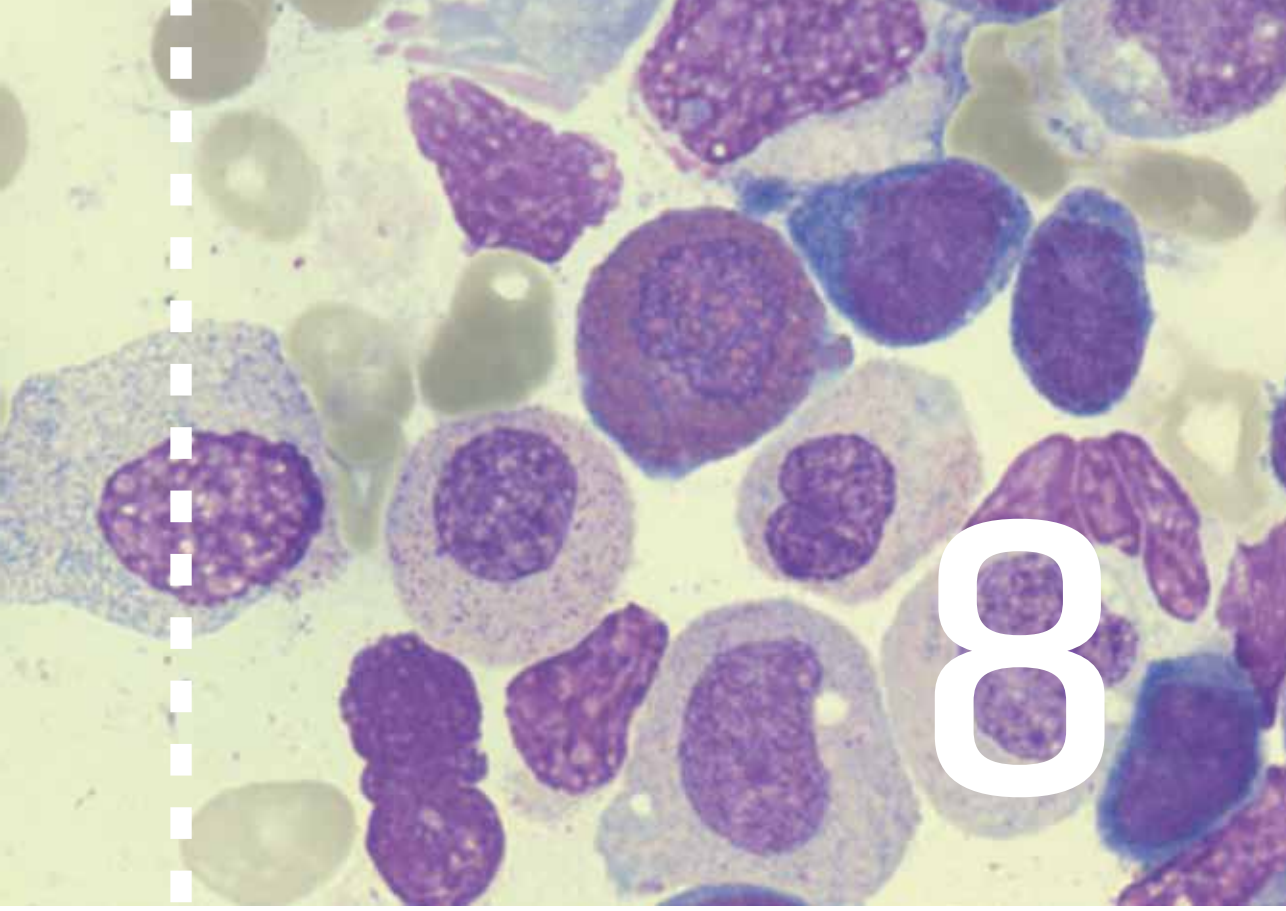
REFERENCES:

1. Hasle H, Niemeyer CM, Chessells JM, Baumann I, Bennett JM, Kerndrup G, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia. 2003 Feb;17(2):277-82.
2. EWOG-MDS. Clinical Trial Protocol EWOG-MDS 2006. Prospective non-randomized multi-center study for epidemiology and characterization of Myelodysplastic Syndromes (MDS) and Juvenile Myelomonocytic Leukemia (JMML) in childhood. 2007.
3. Kardos G, Baumann I, Passmore SJ, Locatelli F, Hasle H, Schultz KR, et al. Refractory anemia in childhood: a retrospective analysis of 67 patients with particular reference to monosomy 7. Blood. 2003 Sep 15;102(6):1997-2003.
4. Gohring G, Michalova K, Beverloo HB, Betts D, Harbott J, Haas OA, et al. Complex karyotype newly defined: the strongest prognostic factor in advanced childhood myelodysplastic syndrome. Blood. 2010 Nov 11;116(19):3766-9.
5. Steensma DP, Neiger JD, Porcher JC, Keats JJ, Bergsagel PL, Dennis TR, et al. Rearrangements and amplification of IER3 (IEX-1) represent a novel and recurrent molecular abnormality in myelodysplastic syndromes. Cancer Res. 2009 Oct 1;69(19):7518-23.
6. Prall WC, Czibere A, Grall F, Spentzos D, Steidl U, Giagounidis AA, et al. Differential gene expression of bone marrow-derived CD34+ cells is associated with survival of patients suffering from myelodysplastic syndrome. International journal of hematology. 2009 Mar;89(2):173-87.
7. Tissing WJ, Meijerink JP, Brinkhof B, Broekhuis MJ, Menezes RX, den Boer ML, et al. Glucocorticoid-induced glucocorticoid-receptor expression and promoter usage is not linked to glucocorticoid resistance in childhood ALL. Blood. 2006 Aug 1;108(3):1045-9.
8. Stam RW, den Boer ML, Meijerink JP, Ebus ME, Peters GJ, Noordhuis P, et al. Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood. 2003 Feb 15;101(4):1270-6.
9. Balgobind BV, Van Vlierberghe P, van den Ouweland AM, Beverloo HB, Terlouw-Kromosoeto JN, van Wering ER, et al. Leukemia-associated NF1 inactivation in patients with pediatric T-ALL and AML lacking evidence for neurofibromatosis. Blood. 2008 Apr 15;111(8):4322-8.
10. Van Vlierberghe P, van Grotel M, Beverloo HB, Lee C, Helgason T, Buijs-Gladdines J, et al. The cryptic chromosomal deletion del(11)(p12p13) as a new activation mechanism of LMO2 in pediatric T-cell acute lymphoblastic leukemia. Blood. 2006 Nov 15;108(10):3520-9.
11. Van Vlierberghe P, van Grotel M, Tchinda J, Lee C, Beverloo HB, van der Spek PJ, et al. The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia. Blood. 2008 May 1;111(9):4668-80.
12. Kuipers JE, Coenen EA, Balgobind BV, Stary J, Baruchel A, de Haas V, et al. High IGSF4 expression in pediatric M5 acute myeloid leukemia with t(9;11)(p22;q23). Blood. 2011 Jan 20;117(3):928-35.
13. Kalbfleisch J, Prentice R. The statistical analysis of failure time data (ed 2nd): John Wiley 2002.
14. Hofmann WK, de Vos S, Komor M, Hoelzer D, Wachsman W, Koeffler HP. Characterization of gene expression of CD34+ cells from normal and myelodysplastic bone marrow. Blood. 2002 Nov 15;100(10):3553-60.
15. Wu MX. Roles of the stress-induced gene IEX-1 in regulation of cell death and oncogenesis. Apoptosis. 2003 Jan;8(1):11-8.
16. Garcia J, Ye Y, Arranz V, Letourneux C, Pezeron G, Porteu F. IEX-1: a new ERK substrate involved in

both ERK survival activity and ERK activation. EMBO J. 2002 Oct 1;21(19):5151-63.

17. Shen L, Zhi L, Hu W, Wu MX. IEX-1 targets mitochondrial F1Fo-ATPase inhibitor for degradation. Cell Death Differ. 2009 Apr;16(4):603-12.

18. Yamashita K, Nakashima S, You F, Hayashi S, Iwama T. Overexpression of immediate early gene X-1 (IEX-1) enhances gamma-radiation-induced apoptosis of human glioma cell line, U87-MG. Neuropathology. 2009 Feb;29(1):20-4.



8

MITOCHONDRIAL DNA MUTATIONS ARE INVOLVED IN THE DEVELOPMENT OF CHILDHOOD MYELODYSPLASTIC SYNDROME

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Submitted

Illustration: MDS, hypogranulation and nuclear-cytoplasm-asynchrony in myelopoiesis, Chediak-Higashi-like granulation (BM)

ABSTRACT

Introduction: Normal cells contain different amounts of mitochondria which can simultaneously harbour wildtype and mutated mitochondrial DNA (mtDNA). Somatic mtDNA variants (either heteroplasmic or homoplasmic) have been found in 50-60% of adult MDS patients, with an increasing frequency with rising age and linearly rising incidences with advanced type MDS. No information is available on the frequency and role of mtDNA variants in pediatric MDS. We recently identified a pediatric MDS case in a family with (germline) mtDNA variants, and investigated whether and how often mtDNA variants occur in an extended cohort of childhood MDS.

Methods: We analysed the role of mtDNA variants in the index family, and in 19 childhood MDS patients, including primary, therapy-related and familial MDS, using the Mito-Chip2 (Affymetrix). The mitochondrial variants were validated by direct sequencing. Variants in the mtDNA were defined as a frequency of less than 1% in the normal population in the different mitochondrial databases. A pathogenicity score was calculated of all found variants. To investigate whether the variants were germline or somatic, analysis was performed on DNA extracted from cultered fibroblasts or from saliva in MDS patients and their mothers. Oxidative phosphorylation assays were performed in the index family to evaluate the enzyme activity of the complexes.

Results: In 13/19 (68%) pediatric MDS patients non-recurrent mtDNA variants were found versus 0.5% in the controls. Seven variants were scored as potential pathogenic. Mt-mutational status did not point towards a specific WHO subgroup of childhood MDS. Heteroplasmic variations were only found as somatic events, whereas germline variants were solely homoplasmic. Nine of the thirteen patients with mtDNA variants carried either additional cytogenetic or molecular aberrations.

Conclusion: We describe the first family in which germline mtDNA variants triggered the development of pediatric MDS and show that also in non-familial pediatric MDS cases, somatic and germline variations frequently occur. This illustrates that mtDNA variants may cause an increased vulnerability for additional hits involved in the development of pediatric MDS.

INTRODUCTION

Myelodysplastic syndrome (MDS) is a clonal stem cell disorder reflected by somatic cytogenetic abnormalities found in early hematopoietic progenitors (1). MDS is rare in childhood, accounting for less than 5% of all hematological malignancies (2). Until now, only scarce information is available on the pathogenesis of pediatric MDS.

Studies in adults have shown that different genetic and molecular events contribute to the development of MDS (3, 4). Alternatively, some studies have emphasized that an auto-immune attack on the stem cells may initiate the disease (5, 6). In addition, somatic mitochondrial DNA (mtDNA) variations have been shown to be involved in the pathogenesis of the disease in adults (7). So far, no information is available on the role of mtDNA variations in sporadic or familiar childhood MDS patients.

MtDNA variations are maternally inherited (8, 9), but can occur *de novo*. MtDNA has no protective histones and no effective repair system. Moreover, the mtDNA is highly exposed to oxygen free radicals, generated by the respiratory chain, as it is located near the inner membrane of the mitochondria. This results in a higher mutation rate than that of chromosomal DNA (10). In general, cells contain different amounts of mitochondria which can harbour both wildtype and variant mtDNA. Co-existence of normal and variant mtDNA is referred to as heteroplasmy, whereas the existence of only normal or only variant mtDNA is called homoplasmy.

MtDNA consists of a hypervariable control region (D-loop, basepair 1-576 and 16024-16569), which regulates the replication and transcription of other mtDNA genes. Sharawat *et al* showed that aberrations in the D-loop of the mtDNA occur in a high frequency in pediatric acute myeloid leukemia (AML) and that specific aberrations were associated with an inferior event-free survival (11). Apart from the hypervariable D-loop, the mtDNA includes a coding region (basepair 577-16023), which encodes for 2 ribosomal genes and 22 tRNAs that are essential for translating the RNA of the protein encoding genes. The coding part also encodes for 13 proteins of the oxidative phosphorylation enzyme complexes: ND1-6 and ND4L (Complex I), CO1-3 (Complex III), Cytochrome B (Complex IV) and ATPase 6 and 8 (Complex V). Variations in the genes encoding for the proteins of the oxidative phosphorylation can result in an aberrant function of the complex, leading to impaired energy production. It is hypothesized that this impaired energy production results in an abnormal function of the spindle apparatus, necessary for cell division, which subsequently leads to an increased chromosomal instability (12). Somatic mtDNA variations contribute to the development of adult MDS/AML as well as childhood AML (7, 11). However, MDS susceptibility in pediatric cases based on inherited mtDNA variations has never been reported. In this paper we describe an index childhood MDS case with germline mtDNA variants and with affected family members, followed by investigating the occurrence of germline and somatic mtDNA variants in a serie of childhood MDS cases.

PATIENTS AND METHODS

Index family

In the index family, in which one young girl had been diagnosed with a bilateral optical nerve atrophy associated with 2 germline mitochondrial mutations (i.e m.13528 A>G and m.13565 C>T), two sisters and the mother were diagnosed with MDS.

Additional pediatric MDS cohort

For additional mutation analysis screening 17 sporadic pediatric MDS patients, previously diagnosed in our hospital with MDS, and 2 familial pediatric MDS cases were included. Informed consent from the parents and children was obtained according local law and regulations.

Controls

For the evaluation of mitochondrial variants in MDS patients, data were compared with the population frequency described in the Human Mitochondrial Genome Database (mtDB, www.genpat.uu.se) (13) as well as mtSNP (www.mtsnp.tmig.or.jp/mtsnp/index_e.shtml) and mitomap (www.mitomap.org). At the moment, these databases contain 2704 published mitochondrial genome sequences (13). Aberrations occurring in more than 1% of the healthy population described in the mtDB are defined as single nucleotide polymorphisms (SNPs) (13). An aberration was defined as a variation when the incidence of this aberration was less than 1% in the healthy population described in the mtDB. As previously defined, the variation was called a mutation when it is a potential pathogenic aberration, based on the pathogenicity score, which is calculated for the variations according to a checklist (14-16). The variation frequency in the MDS cohort was compared to a healthy control cohort in which recently mtDNA variations were found with a frequency of 1/200 (26).

Isolation of DNA

Mononuclear cells were derived from cryopreserved primary bone marrow at diagnosis (17). Cells were thawed and resuspended in RMPI 1640 medium (Dutch modification without L-glutamine; 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, 0,125 µg/ml fungizone and 0,2 mg/ml gentamycin (Gibco BRL, Life Technologies, Breda, The Netherlands). Genomic DNA was extracted from a minimum of 5 x 10^6 cells using TRIzol reagent (Gibco BRL, Life Technologies, Breda, The Netherlands) according to the manufacturer's instructions.

Mitochondrial DNA mutation analysis

The entire mtDNA was amplified using the Expand Long Template system (Roche, Mannheim, Germany) in two fragments. Equimolar amounts of the amplified fragments were pooled and fragmented together with amplified control sequence, as previously described (18). Thereafter, DNA was labeled and hybridized on the MitoChip2 (Affymetrix, Santa Clara, CA, USA). The MitoChip2 contains both sense and antisense probes specific for the forward and reverse sequence of the entire mtDNA. Oligonucleotide probes were synthesized in situ on the Affymetrix chip by standard photolithography and solid-

phase DNA synthesis. For each position of the mtDNA fragment four 25-mer probes are represented on the chip, each with a different nucleotide in the middle allowing the detection of all possible nucleotide substitutions. After washing and staining the MitoChips were scanned using the Affymetrix GeneChip scanner 3000 creating CEL files, which were analysed with Affymetrix GeneChip DNA Analysis Software (18, 19).

Validation of mitochondrial mutations by direct sequencing

After MitoChip analysis, identified variants were confirmed by conventional sequencing using the BigDye terminator v3.1 Cycle sequencing kit, an ABI-PRISM 3100 genetic analyser and the Sequence Analysis 3.7 software package (18).

For analysis of the germline material the RSS000056015_01 mitoSEQr™ Resequencing Primers for the complete human mitochondrial genome of Applied Biosystems were used (Applied Biosystems, Foster City, California, USA). For the PCR reaction 5.0 µL AmpliTaq GoldR PCR Master Mix (2X), 1.6 µL 50% of UltraPure™ glycerol, 1.0µL (0.6 µM/µL) Forward mitoSEQr™ RSA primer, 1.0µL (0.6 µM/µL) Reverse mitoSEQr RSA primer and 1.0 µL (0.5–1.0 ng/µL) genomic DNA were mixed. The thermocycling conditions with the AB 9700 are 5 min heat activation at 96°C, followed by 40 cycles of 94°C for 30 sec, 60°C for 45 sec, and 72°C for 45 sec. Final extension of 72°C for 10 min and hold at 4°C. The PCR reaction product was cleaned up by adding 2 µL of ExoSAP-ITR (USB Corporation Isogen Life Science, De Meern, The Netherlands), which is incubated at 37°C for 30 min followed by heat inactivation at 80°C for 15 min. For the sequence reaction a forward and reverse sequencing reaction mix was prepared. For 10-µL sequencing reactions 4.0 µL of BigDyeR Terminator Ready Reaction Mix v3.1, 1.0 µL of the M13 forward or reverse primer (3.2 pmol/µL), 3.0 µL of deionized water and 2.0 µL of the cleaned up PCR product were used. Thermocycling conditions with the AB 9700 were, heat activation of 96°C for 1 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min, subsequently held at 4°C. The sequence reaction product was cleaned up by adding 2.5 µL of 125 mM EDTA. After mixing, 30 µL of 100% ethanol was added and incubation was performed at room temperature for 15 min. Thereafter, the mix was centrifuged at 2000 x g for 45 min., after which the supernatant is removed and 30 µL of 70% ethanol was added and centrifuged at 2000 x g for 15 min. and after removing the supernatant and dried on air. After resuspending in 10 µL of Hi-Di™ Formamide, the product is analysed with ABI3100 Genetic Analyzer (Applied Biosystems).

To determine whether the identified variants in the index family were germline or somatic, mtDNA screening was performed on cultered fibroblasts obtained by skin biopsies. In the sporadic MDS patients and four mothers genomic DNA was extracted from saliva by the self-administrated Oragene method (20). Genomic DNA was obtained from collected saliva by using the Qiagen DNA isolation kit, according to the manufacturer's instructions. Quantification of DNA was performed using a spectrophotometer.

Oxidative phosphorylation

The activity of the enzymes involved in the oxidative phosphorylation was measured in fibroblasts of the index family members. Complex I (nicotinamide adenine dinucleotide hydrogenase coenzyme Q reductase) was measured as the rotenone-sensitive oxidation of nicotinamide adenine dinucleotide hydrogenase with decylubiquinone as an electron acceptor, as previously described Complex I activity was expressed as

percentage of the mean value of controls (21). Complex II (succinate-coenzyme Q reductase) was determined as the 2 mM 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione sensitive reduction of 2,6-dichlorophenolindophenol by succinate in the presence of decylubichinon. Complex III (ubiquinol-cytochrome *c* reductase) was measured as the antimycin A sensitive reduction of cytochrome *c* by decylubiquinol. Complex IV (cytochrome *c* oxidase) was determined by following the oxidation of reduced cytochrome *c* in the presence of Tween-20 as detergent. Citrate synthase activity was determined as a marker enzyme for mitochondrial density and measured according to Srere, protein was assayed with the Lowry method. Protein content and enzymatic activities were reported per gram wet weight of tissue sample. Oxidative complex activities were also calculated per unit of citrate synthase activity (22-24).

Additional molecular aberration screening

All patients were screened for molecular aberrations in the following genes: *FLT3-ITD, FLT3-TKD, BRAF, NRAS, KRAS, PTPN11, c-KIT, NPM1, CEBPA, RUNX1, P53, TET2, ASXL1, DNMT3A, IDH1* and *IDH2* as described in chapter 6.

RESULTS

Index family

The pedigree of the index family is indicated in Figure 1. Subject 4 was diagnosed at the age of 15 years with refractory anemia with excess of blasts (MDS-RAEB). Cytogenetic and molecular analysis of the bone marrow revealed: 46, XX, +1, der(1;7)(q10;p10)[4], 47, idem, +11[7], 46, XX[29]) as well as a *FLT3-ITD* mutation. The latter disappeared after one cycle of intensive chemotherapy, which was started because of progression to AML, before stem cell transplantation. Family donor search revealed non-transfusion dependent MDS-RCC (refractory cytopenia of childhood) (bilineage dysplasia without an increased percentage of blasts) with trisomy 8, both in the twin sister (subject 5) and the mother (subject 1), which remained stable until today without intervention (follow-up time 2 years). Mutational screening of subject 1, 4 and 5 did not reveal any molecular aberrations of the *FLT3-TKD, PTPN11, RAS, BRAF, c-KIT, CEPBA, NPM1, TET2, ASXL1, P53* or *RUNX1* gene. Peripheral blood counts and differentiation were normal in the older sister (age 17 years old) who was diagnosed with a mtDNA mutation related bilateral optic nerve atrophy at the age of 4 years (subject 3, Table 1). For the purpose of donor screening, mutation analysis of germline DNA of fibroblasts of subject 3 and bone marrow and fibroblasts of all three subjects with MDS (subjects 1, 4 and 5) was performed, which revealed two similar homoplasmic mtDNA mutations (m.13528 A>G (T398A) and m.13565 C>T (S410F)) as previously found in the eldest sister (subject 3). The region in which these mutations occurred is encoding for the *ND5* gene, which encodes for one of the subunits of the NADH dehydrogenase (complex I). This enzyme complex of the oxidative phosphorylation catalyzes the electron transfer from NADH to coenzyme Q (25). Oxidative phosphorylation screening showed decreased activity of the enzymes of complex I (muscle cells in subject 3 and fibroblasts in subject 1, 3, 4 and 5), possibly related to the mutations in the *ND5* gene (Table 1).

Table 1: Patient characteristics of index family with three MDS patients.

	1 (mother)	2 (father)	3 (child 1)	4 (child 2, index patient)	5 (child3)
Diagnosis	MDS refractory anemia	no MDS	Visual disabled	MDS RAEB	MDS RCC
Blood cell count					
Hb (mmol/l)	7.3	9.6	8.4	6.5	9.0
WBC (x 10^9/l)	3.8	13.8	5.5	1.3	3.3
ANC (x 10^9/l)	3.1	10.2	3.2	0.1	0.78
Plt (x 10^9/l)	173	262	243	23	161
Blast (%)	0	0	0	2	0
Bone marrow					
Blast (%)	0	0	0	11	0
Dysplasia	dysmyelopoiesis and dysmegakarypoiesis	no dysplasia	no dysplasia	dysmyelopoiesis and dysmegakaryopoiesis	dysmyelopoiesis and dysmegakarypoiesis
Cytogenetics at diagnosis	47, XX, +8 [2]/46, XX[19]	46, XY	46, XX	46, XX, +1, der (1;7) (q10;p10)[4], 47, idem, +11[7], 46, XX[29]	47, XX,+8 [9], 46, XX [28]
Molecular aberration	none	none	none	FLT3 ITD	none
mtDNA mutation	m.13528 A>G m.13565 C>T	no mutations	m.13528 A>G m.13565 C>T	m.13528 A>G m.13565 C>T	m.13528 A>G m.13565 C>T
Enzyme activity complex I in fibroblasts (amount of substrate / (min U citratesynthesis), (% compared to controls)	0.09 (48%, decreased)	normal	0.14 (73%, slightly decreased, 57% in muscle, decreased)	0.11 (58%, decreased)	0.13 (67%, decreased)

Hb = hemoglobin, WBC = white blood cell count, ANC = absolute neutrophile count, Plt=platelets, RAEB = refractory anemia with excess of blasts, RCC = refractory cytopenia of childhood

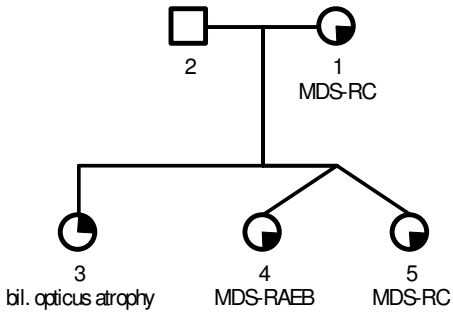


Figure 1: Pedigree of the index family. RC= refractory cytopenia, RAEB= refractory anemia with excess of blasts.

Cohort of additional pediatric MDS cases

MtDNA mutation analysis in an additional serie of 17 sporadic childhood MDS cases from our institute (6 RCC, 6 RAEB, 2 RAEB-t, 1 RARS, and 2 secondary MDS cases) and 2 familial cases revealed 18 variations in 13/19 patients, which will be described in detail (Table 2, Figure 2). All variations were found in the sporadic MDS cases.

MtDNA variations in the hypervariable control region (D-loop)

All 19 patients revealed variations in the control region genes (basepair 1-576 and 16024-16569). A total of 143 variations, confirmed by sequencing, were found at 70 different positions in the D-loop, with a median of eight variations per patient (range: 2-12) (Table 3A). Six of the 143 variations were not described before in the known databases and 21/143 were found in less than 1 percent in healthy controls described in the known mitochondrial DNA databases (Table 3A). The number of patients was to small to correlate the varants with outcome.

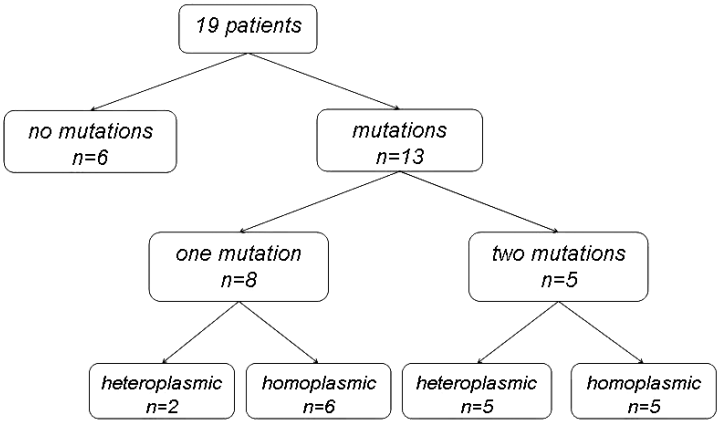


Figure 2: MtDNA variants in 19 MDS patients.

MtDNA variants in the coding genes

The frequency of mtDNA variants in the coding genes (consisting of ribosomal RNA genes, tRNA genes and genes encoding for proteins of the complexes of the oxidative phosphorylation) was 13/19 (68%) as compared to 1/200 in healthy controls (0.5%) (26). These included single mtDNA variant in 8 patients (6 homoplasmic mutations, 2 heteroplasmic) and two concommittant mtDNA variants (5 homoplasmic and 5 heteroplasmic mutations) in 5 patients (Figure 2). Of the 18 identified variants, 7 affected protein function, based on the pathogenicity score (14-16). All detected variants are shown in Table 2.

In total five, all novel, ribosomal RNA gene (MT-RNR1 = 12S rRNA, and MT-RNR2 = 16S rRNA) variants were observed in the mitochondrial genomes of the 19 analyzed MDS patients. One patient carried two mutations in the ribosomal RNAs. Three of the mutations were heteroplasmic (m.989T>Y, m.1952T>Y and m.3199T>Y) and two homoplasmic (m.2757A>G and m.2879A>G).

Two of the nineteen subjects carried mutations in the tRNA genes (Figure 3). One, m.8286A>G (homoplasmic mutation), maps in the tRNA lysine (MT-TK) and is located in the acceptor stem of the tRNA (Figure 3A). The other mutation m.12317 T>Y (heteroplasmic mutation) which is mapping in the tRNA Leucine (MT-TL2), results in a mismatch in the TΨC loop stem and has not been described previously (Figure 3B).

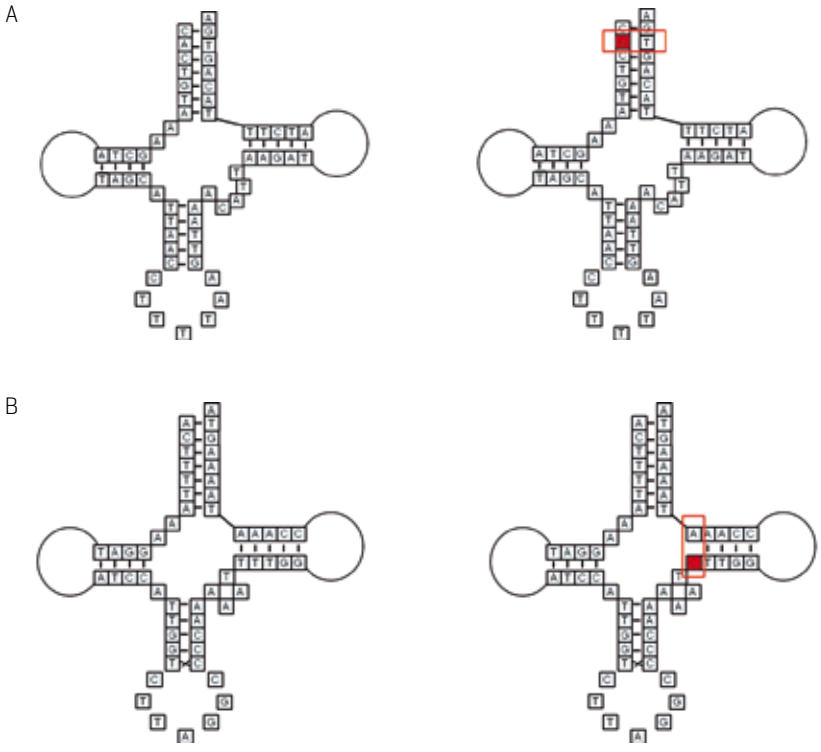


Figure 3: Mutations in the tRNA. Figure 3A: tRNA lysine. On the left site the wildtype tRNA lysine, on the right site the tRNA lysine with m.8296 A>G. Figure 3B: tRNA leucine. On the left site the wildtype tRNA leucine, on the right sight the tRNA leucine with m.12317T>Y

Table 2: Patient characteristics additional MDS cohort.

Pt.	Diagnosis	Age at diagnosis	Gender	Blast (%)	Cytogenetics	Mt DNA mutation	Affected gene	Affected complex	Amino acid substitution
A	Sec. MDS	16	M	0	46, XY	m.7278T>Y m.8568C>A	CO I ATPase8	Complex III Complex V	F36L S68Y
B	RAEB	5	M	7	45,XY,r(6)[p?q?],7,t[17;22][q1?1;q11] c[33]/46,XY,t[17;22][q1?1;q11]c[4]	m.8296A>G	tRNA lysine		-
C	RARS	14	M	1.4	46,XY,idic[6](p2??2q?)[17]/47,idem,+idic[6][4]/45,XY,-6[5]	m.7692T>C m.14319T>C	CO II ND6	Complex III Complex I	F36S N119D
D	RCC	12	F	2.2	45, XX,-7[31]	m.11253T>C m.11778G>R	ND4 ND4	Complex I Complex I	I165T R340H
E	RCC	8	M	0	46, XY	m.14751C>T	Cyt B	Complex IV	T2I
F	RAEB-T	2	M	7.8	43-50,Y,-X,-1,-2[3],del(5)[q11q13][18],+6[15], -9 [3]der (10)t(1;10)[p13;q26), -13[18],-18[3], -19[13], add(19)[p11) [8], +21[16]+21[14],+mar1[18], +mar2[11],+mar3[19], +ring[2] [cp20]	no aberration	-		-
G	RCC	13	M	6.6	46, XY	m.3199T>Y	16s rRNA		-
H	RAEB-T	14	F	22	46, XX	m.989T>Y m.2757A>G	12s rRNA 16s rRNA		- -
I	RAEB	12	M	7.6	45, XY, -7[22]	no aberration	-		-
J	RAEB	16	M	11.6	46, XY, der(1)add(1)(p3?5)add(1)(q31~q32), der(10)t(1;10)(q22;q26), add(1)(q13)[19]/46, XY [1]	m.1952 T>Y m.12317T>Y	16s rRNA tRNA leucine		- -
K	RCC	10	M	2	46, XY	no aberration	-		-
L	RCC	5	M	0.4	46, XY	m.12338T>C	ND5	Complex I	M1T
M	RAEB	12	F	3.9	47, XX, +1, der(1;7)(q10;p10), +8[15]	m.14954A>G	Cyt B	Complex IV	T70A
N	Fam MDS: RCC	2	M	0	46, XY	no aberration	-		-
O	Fam MDS: RCC	3	F	0	46, XX	no aberration	-		-
P	RAEB	17	F	11.2	47, XX, +8 [13]/46 XX[9]	no aberration	-		-
Q	Sec. MDR-AML	8	F	21	46, XX, -7, i(21)(q10)[15]	m.12557C>T	ND5	Complex I	T74I
R	RCC	3	F	0	45, XX, -7[8]/46, XX[4]	m.10750A>R	ND4L	Complex I	N94S
S	RAEB	8	M	0	45, XY, ring 6, -7	m.2879A>G	16s rRNA		-

Sec. MDS = Secundary myelodysplastic syndrome, RAEB = refractory anemia with excess of blasts, RARS = refractory anemia with ringsideroblasts, RCC=refractory cytopenia of childhood, Fam. MDS = familial myelodysplastic syndrome, MDR-AML = MDS related acute myeloid leukemia, M = male, F = female, IVIG = intraveneous immunoglobulins, IST = immunosuppressive therapy, Haplo-id SCT = haplo-identical stem cell transplantation, TRM = therapy related mortality

	Pathogenecity	Germline analyses	Analysis mother	Frequency healthy popultaion	Treatment	Outcome	F ollow up (years)
	affect protein function affect protein function	NA		2/2704 0/2704	MUD-SCT	alive	6
		NA		4/2704	MUD-SCT	alive	10
	affect protein function affect protein function	NA		0/2704 1/2704	MUD-SCT	died, TRM	2
	tolerated affect protein function	NA		10/2704 2/2704	MUD-SCT	died, TRM	0,8
	affect protein function	m.14751C>T	wild type	2/2704	IVIG	alive, relapse after 6.33 years	7
		NA			AML-Rx, MUD-SCT	died, relapse after 0,6 years	0,8
		wild type		0/2704	MUD-SCT	alive	7
		wild type m.2757A>G	wildtype m.2757A>G	0/2704 3/2704	Chemo, MUD-SCT	alive	7
		NA			MSD-SCT	died, TRM	0,5
		NA		0/2704 0/2704	MUD-SCT	died, TRM	0,5
		NA			IST, IVIG, MUD-SCT	alive	7
	affect protein function	NA		0/2704	IST, haplo-id SCT	graft failure, retransplant, died TRM	1,5
	tolerated	m.14954A>G	m.14954A>G	1/2704	MUD-SCT	alive	2
		NA			SCT	died TRM	0.5
		NA			supportive care	alive	3
		NA			haplo-id SCT	alive	1,5
	tolerated	NA		4/2704	chemo	died, TRM	0,2
	tolerated	wild type	wild type	7/2704	MUD-SCT	alive	11
		NA		0/2704	Chemo	died before transplantation due to progression disease	1,5

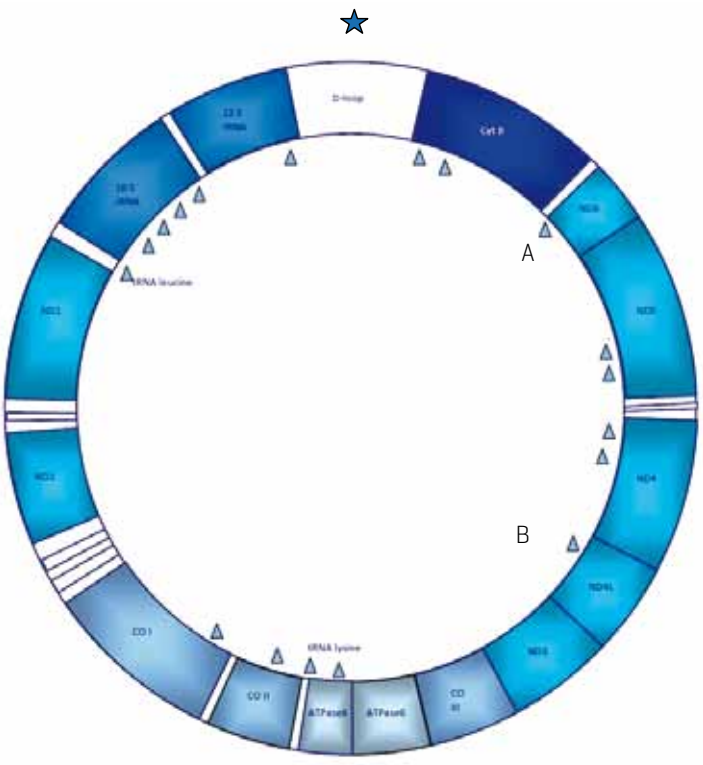
Table 3A: Sites of aberrations in control region (D-loop) of the mtDNA in 19 patients.

Position of nucleotide	Nucleotide variation	Number of patients with aberration
72	T>C	1
73	A>G	12
146**	T>C	1
150	C>T	2
152	T>C	5
195	T>C	4
199	T>C	1
204*	T>A	1
207	G>A	1
217	T>C	1
239**	T>C	1
242**	C>T	1
263	A>G	15
285**	C>T	1
295	C>T	1
321*	T>C	1
385**	A>G	1
461*	C>T	1
462	C>T	1
471**	T>C	1
477**	T>C	1
489	T>C	3
497	C>T	1
499	G>A	1
508**	A>G	1
511**	C>T	1
15954**	A>C	1
16051	A>G	2
16069	C>T	1
16093	T>C	2
16104*	C>T	1
16111**	C>T	1
16126	T>C	3
16129	G>A	2
16129**	G>C	1
16140**	T>C	1

Table 3A: Sites of aberrations in control region (D-loop) of the mtDNA in 19 patients.

Position of nucleotide	Nucleotide variation	Number of patients with aberration
16145	G>A	1
16172	T>C	1
16183	A>C	3
16189	T>C	4
16192	C>T	3
16213**	G>A	1
16221**	C>T	1
16222**	C>T	1
16223	C>T	4
16224	T>C	1
16227	A>G	1
16234	C>T	1
16249	T>C	1
16256	C>T	2
16257**	C>T	1
16261	C>T	1
16263**	T>C	1
16270	C>T	3
16278	C>T	1
16288	T>C	1
16289*	A>G	1
16291	C>T	1
16292	C>T	1
16294	C>T	3
16296*	C>G	1
16304	T>C	2
16311*	T>C	1
16319	G>A	1
16356	T>C	1
16362	T>C	5
16398**	G>A	1
16399	A>G	2
16428**	G>A	1
16482**	A>G	1
16519	T>C	12

*= not described previously in the known mtDNA databases
**= found in less than 1% in the known mtDNA databases



★ Aberrations in D-loop described in table 3A

Table 3B: Distribution of the mtDNA variants.
ND1= NADH dehydrogenase subunit 1, ND2= NADH dehydrogenase subunit 2, ND3= NADH dehydrogenase subunit 3,ND4= NADH dehydrogenase subunit 4, ND5= NADH dehydrogenase subunit 5, ND6= NADH dehydrogenase subunit 6, CO I= cytochrome c oxidase subunit 1, CO II= cytochrome c oxidase subunit 2, CO III= cytochrome c oxidase subunit 3, cytB= cytochrome b.

Eleven out of the 19 mtDNA genomes of the MDS patients showed variants in the protein coding regions (8 homoplasmic and 3 were heteroplasmic) (Table 2 and 3B). Six of these eleven variants were found in complex I: a heteroplasmic variant in the *ND4L* gene (NADH dehydrogenase subunit 4L) in one patient, two variants in the *ND4* gene (NADH dehydrogenase subunit 4) (one homoplasmic and one heteroplasmic) in one patient, two patients with homoplasmic variants in the *ND5* gene (NADH dehydrogenase subunit 5) and in one patient a homoplasmic variation in the *ND6* gene (NADH dehydrogenase subunit 6). Two of the identified variations encode for proteins of complex III: one heteroplasmic variation in the *CO-I* gene (cytochrome c oxidase subunit I and a homoplasmic variation in the *COII gene* (cytochrome c oxidase subunit II). Two variations were found in genes encoding for proteins of complex IV: both homoplasmic variants in the *cytochrome B* gene in two different patients. Furthermore, one patient carried a homoplasmic variant in the ATPase8 (complex V).

Analysis of germline mtDNA mutations

In 5 of the 13 sporadic MDS cases with variants in coding mtDNA genes (3 homoplasmic and 2 heteroplamic variants), germline material was available for analysis (patient E, G, H, M and R). The presence of homoplasmic variants m.14751C>T (patient E), m.2757A>G (patient H, Figure 4A) and m.14954A>G (patient M) were confirmed in germline material. In contrast, the heteroplasmic variants m.989T>Y (patient H, Figure 4B), m.3199T>Y (patient G) and m.10750A>R (patient R) were not detected in saliva and were therefore considered as somatic variants (Table 2).

Analysis of maternal samples

In 4 of the 13 patients with a mtDNA variant it was possible to study maternal saliva. In case of the 2 homoplasmic variants we could confirm that these variants were inherited from the mother. The 2 heteroplasmic variants were not found in the germline DNA of their mothers (Table 2).

Correlation mtDNA variants and cytogenetic and molecular aberrations

Five of the thirteen patients with a mtDNA variant in coding genes and 3/6 patients without mtDNA variants carried a normal karyotype. Extensive molecular analysis of the patients with mtDNA variants revealed only one additional germline *RUNX1* mutation (patient H) and an additional somatic *PTPN11* mutation (patient S).

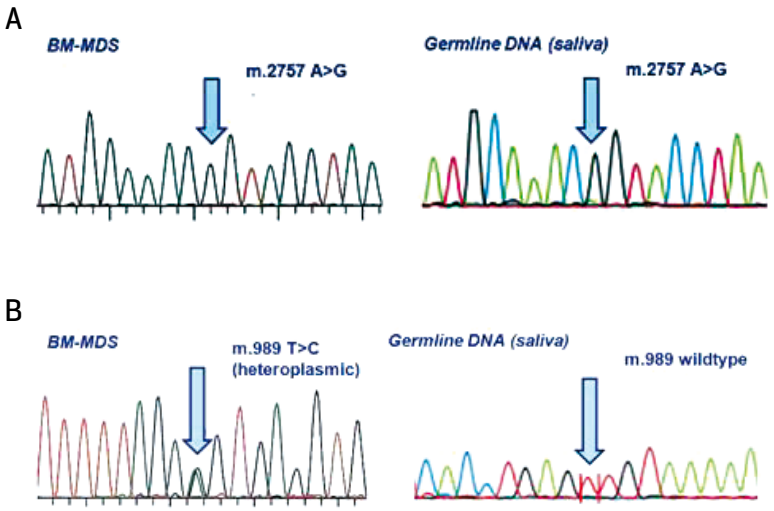


Figure 4: Comparison mtDNA variants in bone marrow with germline material. Figure 3A: Homoplasmic variant m.2757A>G. The homoplasmic mtDNA variant m.2757A>G is found in the bone marrow at diagnosis of the myelodysplastic syndrome and the germline material, indicating that this is a germline variant. Figure 3B: Heteroplasmic variant m.989T>C. The heteroplasmic mtDNA variant m.989T>C is found in the bone marrow at diagnosis of the myelodysplastic syndrome and not in the germline material, indicating that this is a somatic variant.

DISCUSSION

Here we describe the first family in which three young first-degree-related female members with MDS carry germline mtDNA variants. This family illustrates the clinical variety of the phenotypes that can be triggered by variants in the mtDNA, as all four female family members were found to carry similar homoplasmic mtDNA variants and three of them suffered from MDS, whereas the fourth female sibling had optic symptoms without signs of MDS. This family illustrates the well-known maternal inheritance of mtDNA mutations, which was discovered by Giles *et al* (27), and which we could further confirm by identifying the same mtDNA mutation in their 90-years old grandmother.

This family history also illustrates that germline mitochondrial variants may be the initiator of a multistep driven development of myeloid malignancy, and suggests that mtDNA mutations in the hematopoietic stem cell increases the vulnerability to acquire genetic variants that lead to MDS or AML (10). So far, this vulnerability was mainly attributed to acquired, somatic mitochondrial mutations, except for cases with Pearson syndrome, where exocrine pancreatic dysfynction can be accompanied by sideroblastic anemia and is caused by a germline deletion in the mtDNA (28, 29).

In our index family the mtDNA variants were shown to be germline variants. The subsequent acquired genetic aberrations, t(1;7) and *FLT3-ITD* mutation in subject 4 and trisomy 8 in subject 1 and 5, were required for the evolvement to clinical MDS. Interestingly, only the patient that acquired an additional *FLT3-ITD* mutation showed enhanced proliferation as reflected by disease progression towards MDS related–AML, consistent with the hypothesis as postulated by Gilliland *et al* (5).

We show that, also in sporadic childhood MDS, mtDNA mutations are a frequent occurring event as two third of our cases carried one or more variations in the mtDNA, which is significantly higher as compared to the healthy population, in which mtDNA mutations are rare (0.5%) (26). The frequency of variations in childhood MDS may be comparable or even higher than in adults, in which 56% of the MDS cases carry a mtDNA variation (n=104) (7), although in elderly MDS these variants occur mainly somatic. This indicates that mtDNA variants contribute to and maybe initiate the development of MDS in children.

The variants in our cohort were distributed over the entire mtDNA genome and no identical variants were found. Thirteen variants were found in coding regions, of which seven are predicted to affect protein function by SIFT and PolyPhen, which indicates that not only the incidence of variants in our cohort is relatively high, but also that they are often pathogenic. The majority of variants (11/18) were found in genes encoding for proteins of the oxidative phosphorylation, with hotspots in genes encoding for proteins of complex I. In addition, in our index family we showed that the variants found in the *ND5* gene resulted in a decreased enzyme activity of complex I, suggesting that variants in the oxidative phosphorylation, and especially in complex I, may play a role in the pathogenesis of childhood MDS. Five variants were located in the ribosomal RNAs, which play a role in the mitochondrial protein synthesis (30) and two variants were found in the tRNAs. These tRNAs are small RNAs, transfering the amino acid leucine or lycine respectively, to a growing polypeptide chain at the ribosome site of the protein synthesis during translation (25). Both variants interrupt the conserved Watson Crick base pair in the acceptor stem (8296A>G) or the TΨC stem of tRNA leucine (12317T>Y), that are

both likely to alter the structure of the tRNA. This altered structure may result in an impaired function of the tRNA and result in aberrant translation.

MtDNA mutations have been found in several types of cancer (12, 31, 32) and the role of mtDNA mutations in the pathogenesis of adult MDS has been described previously (10). MtDNA mutations induce impaired mitochondrial energy production due to ATP deficiency, which results in decreased metabolic activity of the cells. Subsequently, ATP deficiency results in chromosomal instability, as the spindle apparatus necessary for the cell division is ATP dependent. In childhood MDS chromosomal abnormalities frequently occur, i.e. 40% of the primary MDS and 80% of the secondary MDS cases carry an abnormal karyotype (33-35). Here we showed for the first time, that in our index family germline mtDNA variants result in an aberrant enzyme activity of complex I of the oxidative phosphorylation, which, after experiencing different second genetic and molecular hits, induced evolvement to MDS. In the pediatric MDS patient cohort we studied thereafter, we showed that 9/13 patients with mtDNA variants carried additional cytogenetic or molecular aberrations.

Furthermore, our data reveal that germline variants occur in sporadic pediatric MDS. This opens the discussion whether part of sporadic childhood MDS reflects an underlying predisposition to insufficient maturation of the hematopoietic stem cell. This is clinically relevant as it has consequences for counseling and donor choice in this disease that requires hematopoietic stem cell transplantation as the only curative treatment option. Besides this inherited vulnerability, acquired mtDNA mutations may be relevant for the development of childhood MDS. This is underscored by the fact that we detected somatic mutations in the bone marrow of the MDS patients, which could not be confirmed in germline material. Interestingly, the germline mutations all were homoplasmic mutations, whereas the heteroplasmic mutations were all somatic mutations.

All patients in our series showed variations of the D-loop. The D-loop regulates the replication and transcription of mtDNA. Variations in this region have been observed in 20-81% of different solid malignancies (7, 36-40). In 80% of pediatric AML cases variations in the mitochondrial D-loop were found (11). Linnartz *et al* observed that mtDNA variations in the D-loop are responsible for the transformation of normal or dysplastic cells to a leukemic phenotype in adults (41), resulting in increased ROS production which is mutagenic (31).

Most of the detected mtDNA variations in genes coding for proteins in the present study have not previously been described in relation to disease. Some variations have been described in other diseases as epileptic syndromes, cardiomyopathy, and MELAS (m.8296A>G) Leber's hereditary optic neuropathy and progressive dystonia (m.11253T>C, m.11778G>R, m.12338T>C), Parkinson's disease (m.14319T>C) and multiple sclerosis (m.8568C>A) (42). None of our patients have clinical signs of these diseases during or after the MDS period (follow up time till 10 years after diagnosis of MDS). The index family also illustrated that similar mutations can result in different phenotypes, i.e. optic neuropathy in one subject and MDS in other subjects, probably depending of the amount of mutated mitochondria in the tissue.

In conclusion, we describe the first family in which germline mtDNA-mutations, with concomitant aberrant enzyme activity of complex I of the oxidative phosphorylation and a second random genetic and molecular hit, resulted in MDS in 3 young first degree female family members. Progression to AML was only found in the MDS patient with an

additional *FLT3-ITD* mutation supporting the multistep hypothesis of MDS progression to AML (Figure 5). Of high importance is our finding that mtDNA mutations occur in two-third of sporadic childhood MDS cases, and that the homoplastic mutations can be germline vulnerability enhancers of deregulation of the stem cell in at least a part of pediatric MDS cases.

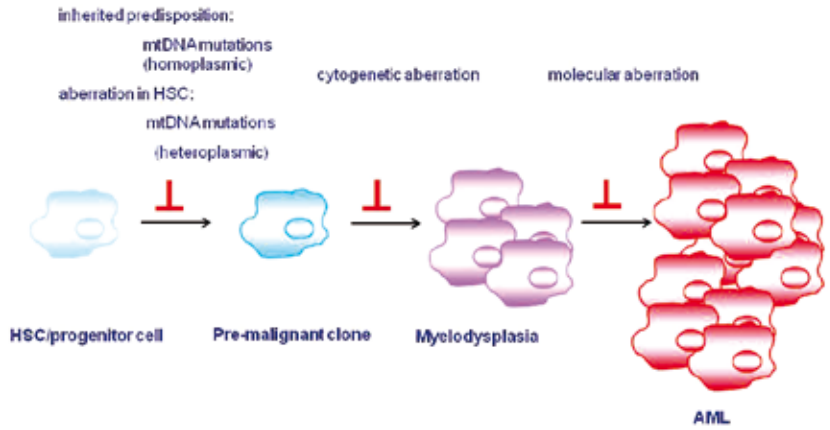


Figure 5: Hypothesis about role mtDNA in the pathogenesis of childhood MDS.

REFERENCES

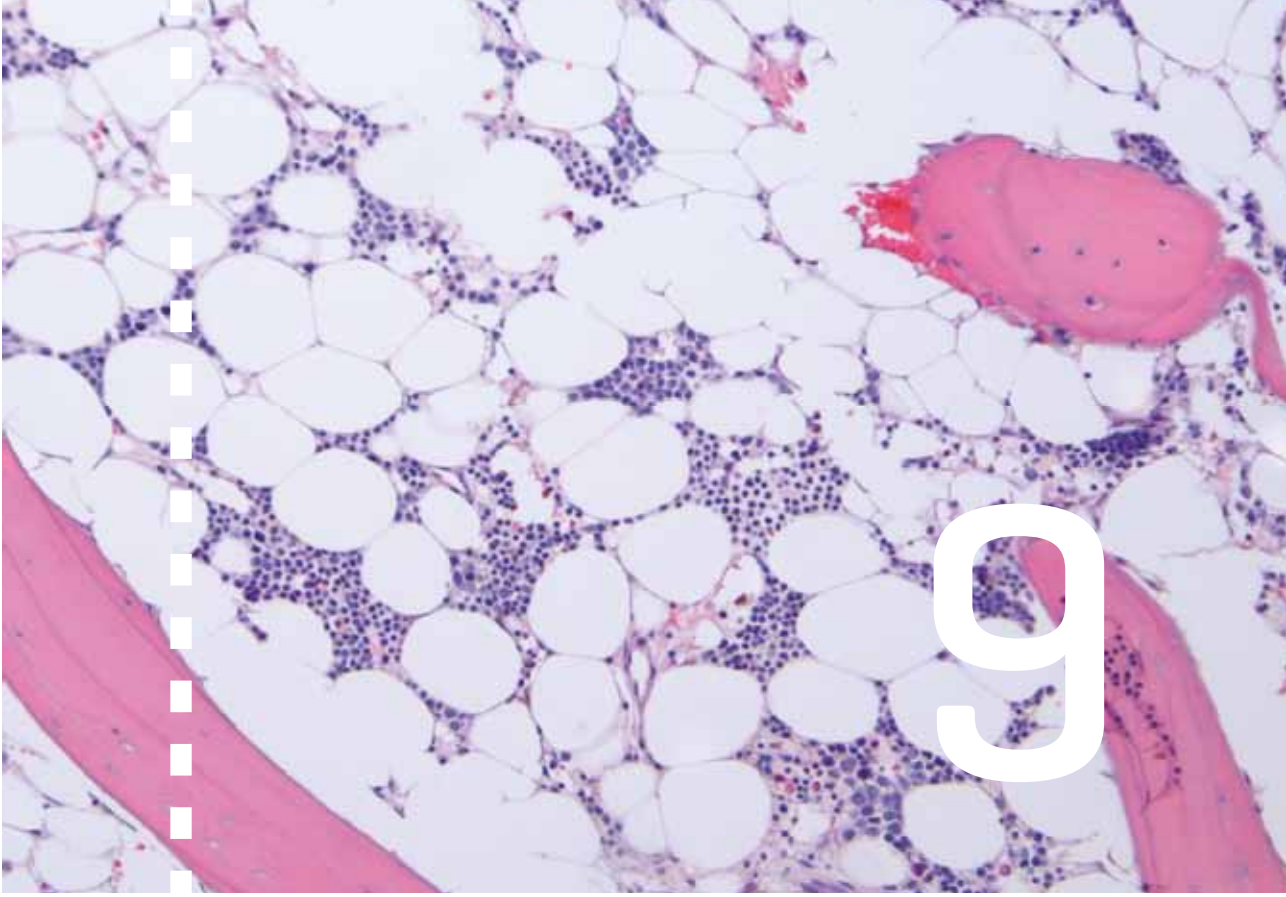
1. Saitoh K, Miura I, Takahashi N, Miura AB. Fluorescence in situ hybridization of progenitor cells obtained by fluorescence-activated cell sorting for the detection of cells affected by chromosome abnormality trisomy 8 in patients with myelodysplastic syndromes. Blood. 1998;92(8):2886-92.
2. Hasle H, Baumann I, Bergstrasser E, Fenu S, Fischer A, Kardos G, et al. The International Prognostic Scoring System (IPSS) for childhood myelodysplastic syndrome (MDS) and juvenile myelomonocytic leukemia (JMML). Leukemia. 2004;18(12):2008-14.
3. Platzbecker U, Meredyth-Stewart M, Ehninger G. The pathogenesis of myelodysplastic syndromes. Cancer Treatment Reviews. 2007:doi:10.1016/j.crtv.2007.07.021.
4. Pedersen-Bjergaard J, Christiansen DH, Desta F, Andersen MK. Alternative genetic pathways and cooperating genetic abnormalities in the pathogenesis of therapy-related myelodysplasia and acute myeloid leukemia. Leukemia. 2006;20(11):1943-9.
5. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet. 2002;3:179-98. Epub 2002/08/27.
6. Deguchi K, Gilliland DG. Cooperativity between mutations in tyrosine kinases and in hematopoietic transcription factors in AML. Leukemia. 2002;16(4):740-4. Epub 2002/04/18.
7. Wulfert M, Kupper AC, Tapprich C, Bottomley SS, Bowen D, Germing U, et al. Analysis of mitochondrial DNA in 104 patients with myelodysplastic syndromes. Experimental hematology. 2008;36(5):577-86.
8. Wallace DC. Mitochondria as chi. Genetics. 2008;179(2):727-35. Epub 2008/06/19.
9. Bellance N, Lestienne P, Rossignol R. Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis. Front Biosci. 2009;14:4015-34. Epub 2009/03/11.
10. Gattermann N. From sideroblastic anemia to the role of mitochondrial DNA mutations in myelodysplastic syndromes. Leukemia research. 2000;24(2):141-51.
11. Sharawat SK, Bakhshi R, Vishnubhatla S, Bakhshi S. Mitochondrial D-loop variations in paediatric acute myeloid leukaemia: a potential prognostic

marker. Br J Haematol. 2010;149(3):391-8. Epub 2010/03/17.

12. Bianchi NO, Bianchi MS, Richard SM. Mitochondrial genome instability in human cancers. Mutation research. 2001;488(1):9-23. Epub 2001/02/27.
13. Ingman M, Gyllensten U. mtDB: Human Mitochondrial Genome Database, a resource for population genetics and medical sciences. Nucleic Acids Res. 2006;34(Database issue):D749-51. Epub 2005/12/31.
14. Nakamura T, Mori T, Tada S, Krajewski W, Rozovskaia T, Wassell R, et al. ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Mol Cell. 2002;10(5):1119-28. Epub 2002/11/28.
15. Tenen DG, Hromas R, Licht JD, Zhang DE. Transcription factors, normal myeloid development, and leukemia. Blood. 1997;90(2):489-519. Epub 1997/07/15.
16. Balgobind BV, Zwaan CM, Pieters R, Van den Heuvel-Eibrink MM. The heterogeneity of pediatric MLL-rearranged acute myeloid leukemia. Leukemia. 2011;25(8):1239-48. Epub 2011/05/14.
17. Gerhardt TM, Schmahl GE, Flotho C, Rath AV, Niemeyer CM. Expression of the Evi-1 gene in haemopoietic cells of children with juvenile myelomonocytic leukaemia and normal donors. Br J Haematol. 1997;99(4):882-7.
18. van Eijsden RG, Gerards M, Eijssen LM, Hendrickx AT, Jongbloed RJ, Wokke JH, et al. Chip-based mtDNA mutation screening enables fast and reliable genetic diagnosis of OXPHOS patients. Genet Med. 2006;8(10):620-7. Epub 2006/11/03.
19. Jacobs L, Gerards M, Chinnery P, Dumoulin J, de Coo I, Geraedts J, et al. mtDNA point mutations are present at various levels of heteroplasmy in human oocytes. Mol Hum Reprod. 2007;13(3):149-54. Epub 2007/01/30.
20. Rylander-Rudqvist T, Hakansson N, Tybring G, Wolk A. Quality and quantity of saliva DNA obtained from the self-administrated oragene method--a pilot study on the cohort of Swedish men. Cancer Epidemiol Biomarkers Prev. 2006;15(9):1742-5. Epub 2006/09/21.
21. De Vries DD, Went LN, Bruyn GW, Scholte HR, Hofstra RM, Bolhuis PA, et al. Genetic and biochemical impairment of mitochondrial complex I activity in a family with Leber hereditary optic neuropathy and hereditary spastic dystonia. Am J Hum Genet. 1996;58(4):703-11. Epub 1996/04/01.
22. Platzbecker U, Santini V, Mufti GJ, Haferlach C, Maciejewski JP, Park S, et al. Update on developments in the diagnosis and prognostic evaluation of patients with myelodysplastic syndromes (MDS): consensus statements and report from an expert workshop. Leukemia research. 2012;36(3):264-70. Epub 2011/12/06.
23. Fenaux P, Giagounidis A, Selleslag D, Beyne-Rauzy O, Mufti G, Mittelman M, et al. A randomized phase 3 study of lenalidomide versus placebo in RBC transfusion-dependent patients with Low-/Intermediate-1-risk myelodysplastic syndromes with del5q. Blood. 2011;118(14):3765-76. Epub 2011/07/15.
24. Trounce IA, Kim YL, Jun AS, Wallace DC. Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. Methods Enzymol. 1996;264:484-509. Epub 1996/01/01.
25. Berg J, Tymoczko J, Stryer L. Biochemistry (6th edition), New York: WH Freeman & Company (2006) pp. 509–513.
26. Elliott HR, Samuels DC, Eden JA, Relton CL, Chinnery PF. Pathogenic mitochondrial DNA mutations are common in the general population. Am J Hum Genet. 2008;83(2):254-60. Epub 2008/08/05.
27. Giles RE, Blanc H, Cann HM, Wallace DC. Maternal inheritance of human mitochondrial DNA. Proc Natl Acad Sci U S A. 1980;77(11):6715-9. Epub 1980/11/01.
28. Pearson HA, Lobel JS, Kocoshis SA, Naiman JL, Windmiller J, Lammi AT, et al. A new syndrome of refractory sideroblastic anemia with vacuolization of marrow precursors and exocrine pancreatic dysfunction. The Journal of pediatrics. 1979;95(6):976-84. Epub 1979/12/01.
29. Rotig A, Colonna M, Bonnefont JP, Blanche S, Fischer A, Saudubray JM, et al. Mitochondrial DNA deletion in Pearson's marrow/pancreas syndrome. Lancet. 1989;1(8643):902-3. Epub 1989/04/22.
30. Noller HF, Green R, Heilek G, Hoffarth V, Huttenhofer A, Joseph S, et al. Structure and function of ribosomal RNA. Biochem Cell Biol. 1995;73(11-12):997-1009. Epub 1995/11/01.
31. Brandon M, Baldi P, Wallace DC. Mitochondrial mutations in cancer. Oncogene. 2006;25(34):4647-62. Epub 2006/08/08.
32. Frezza C, Gottlieb E. Mitochondria in cancer: not just innocent bystanders. Semin Cancer Biol. 2009;19(1):4-11. Epub 2008/12/23.
33. EWOG-MDS. Clinical Trial Protocol EWOG-MDS 2006. Prospective non-randomized multi-center study for epidemiology and characterization of Myelodysplastic Syndromes (MDS) and Juvenile Myelomonocytic Leukemia (JMML) in childhood. 2007.
34. Gohring G, Michalova K, Beverloo HB, Betts D, Harbott J, Haas OA, et al. Complex karyotype newly defined: the strongest prognostic factor in advanced childhood myelodysplastic syndrome. Blood. 2010;116(19):3766-9. Epub 2010/08/31.
35. Kardos G, Baumann I, Passmore SJ, Locatelli F, Hasle H, Schultz KR, et al. Refractory anemia in childhood: a retrospective analysis of 67 patients with particular reference to monosomy 7. Blood. 2003;102(6):1997-2003.
36. Alonso A, Martin P, Albarran C, Aquilera B, Garcia O, Guzman A, et al. Detection of somatic mutations in

the mitochondrial DNA control region of colorectal and gastric tumors by heteroduplex and single-strand conformation analysis. Electrophoresis. 1997;18(5):682-5. Epub 1997/05/01.

37. Ivanova R, Lepage V, Loste MN, Schachter F, Wijnen E, Busson M, et al. Mitochondrial DNA sequence variation in human leukemic cells. Int J Cancer. 1998;76(4):495-8. Epub 1998/05/20.
38. Nomoto S, Yamashita K, Koshikawa K, Nakao A, Sidransky D. Mitochondrial D-loop mutations as clonal markers in multicentric hepatocellular carcinoma and plasma. Clin Cancer Res. 2002;8(2):481-7. Epub 2002/02/13.
39. Tan DJ, Bai RK, Wong LJ. Comprehensive scanning of somatic mitochondrial DNA mutations in breast cancer. Cancer Res. 2002;62(4):972-6. Epub 2002/02/28.
40. Tan DJ, Chang J, Liu LL, Bai RK, Wang YF, Yeh KT, et al. Significance of somatic mutations and content alteration of mitochondrial DNA in esophageal cancer. BMC Cancer. 2006;6:93. Epub 2006/04/20.
41. Linnartz B, Anglmayer R, Zanssen S. Comprehensive scanning of somatic mitochondrial DNA alterations in acute leukemia developing from myelodysplastic syndromes. Cancer Res. 2004;64(6):1966-71. Epub 2004/03/18.
42. Sakuta R, Honzawa S, Murakami N, Goto Y, Nagai T. Atypical MELAS associated with mitochondrial tRNA(Lys) gene A8296G mutation. Pediatr Neurol. 2002



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T-CELL RECEPTOR (TCR) Vβ CDR3 OLIGOCLONALITY FREQUENTLY OCCURS IN CHILDHOOD REFRACTORY CYTOPENIA (MDS-RCC) AND SEVERE APLASTIC ANEMIA

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Illustration: MDS, hypocellular bone marrow (trephine)

ABSTRACT

Background: [Very] severe acquired aplastic anemia [(v]SAA] and myelodysplastic syndrome [MDS] are rare diseases in childhood. [V]SAA is a bone marrow failure syndrome characterized by immune-mediated destruction of hematopoietic progenitors. MDS is a malignant clonal stem cell disorder, of which the hypoplastic variant is, in case of absence of a cytogenetic clone, difficult to separate from [v]SAA. Recently, studies provided a molecular signature of auto-immunity in adult [v]SAA, by showing oligoclonality based on the length of the TCR Vβ CDR3 region. We investigated retrospectively the frequency and the discriminative value of TCR Vβ CDR3 oligoclonality in pediatric [v]SAA and MDS patients.

Methods: Peripheral blood [PB] and/or bone marrow [BM] mononuclear cell samples of pediatric patients with [v]SAA [n=38], refractory cytopenia of childhood [MDS-RCC] [n=28], and 18 controls were analysed via TCR Vβ heteroduplex PCR analysis of extracted RNA.

Results: A skewed TCR Vβ CDR3 repertoire was found in 21/38 [v]SAA and in 17/28 RCC patients in contrast to 2/18 in the control group.

Conclusions: These data suggest an overlapping group of RCC and SAA patients that may share a common immunemediated pathogenesis. Prospective studies are required to establish the clinical value of TCR Vβ CDR3 repertoire analysis to predict the clinical response in these patients.

INTRODUCTION

(Very) severe aplastic anemia (v)SAA and myelodysplastic syndromes (MDS) are rare acquired bone marrow failure disorders in childhood. In the last decade (v)SAA has shown to be associated with autoimmune phenomena (1-2). The use of immunosuppressive therapy (IST) has proven to be of great value in both adults and children with SAA (3-5). The administration of combinations of antithymocyte globulin (ATG), cyclosporine and steroids with or without granulocyte colony-stimulating factor have resulted in an event-free survival of about 70% (4, 6-8).

Myelodysplastic syndrome is a clonal stem cell disorder which results in a dysregulation of hematopoiesis and dysplasia in one or more cell lines. From studies of the European Working Group of MDS and JMML in Childhood (EWOG-MDS) it has become apparent that over 50% of all pediatric MDS are hypoplastic (9). Refractory cytopenia of childhood (RCC) is a subtype of childhood MDS characterized by dysplasia in at least 2 hematopoietic cell lineages, but less than 2% blasts in the peripheral blood (PB), and less than 5% blasts in the bone marrow (BM) (10). In hypocellular cases discriminating RCC from SAA, which is mainly based on morphology and histology, is difficult, especially in case of absence of clonal cytogenetic abnormalities. So far no molecular diagnostic tools are available for characterization and discriminating (v)SAA and MDS-RCC.

In adult (v)SAA the variability of the CDR3 region of the Vβ chain of the T-cell receptor (TCR) has been described as a useful tool to determine an auto-immune patient-specific signature. Moreover, in adult (v)SAA patients the patient-specific oligoclonal pattern has been used to monitor molecular responses (11). This oligoclonality reflects a skewed variability of the CDR3 length of the TCR Vβ chains and therefore is referred to as TCR Vβ skewing (11-14).

In this pilot study, we investigated the frequency of TCR Vβ skewing and the value of molecular TCR Vβ CDR3 repertoire analysis to discriminate childhood v(SAA) from MDS-RCC. Moreover, we analysed whether skewing of individual Vβ families differed between (v)SAA and MDS-RCC. In addition, we evaluated the most informative compartment (PB or BM) to study this oligoclonality.

PATIENTS

In this retrospective study pediatric MDS and (v)SAA patients, diagnosed in pediatric oncology centers and who participated in the EWOG-MDS, were included based on availability of material.

RCC was diagnosed according to EWOG-MDS-98 criteria and (v)SAA according to German SAA 1994 group (Table 1) (4, 10, 15-16). All BM biopsies were centrally reviewed by the morphology board of EWOG-MDS and the German SAA group.

The control group consisted of pediatric patients with Evans syndrome (n=2), congenital bone marrow failure syndrome (n=7), chronic myeloid leukemia (n=1), idiopathic thrombocytopenic purpura (n=2), parvo B19 infection (n=1) and healthy volunteers (n=5). For this retrospective study, clinical characteristics such as gender, age at diagnosis, morphologic subtype, treatment schedule and response to treatment were collected from the medical records in the local hospitals. Approval from local medical ethical committees and written informed consent according the Helsinki agreement were obtained.

Table 1: Diagnostic criteria of MDS-RCC and [v]SAA according to EWOG-MDS-1998 and SAA-1994.

	AA	
MDS-RCC [*]	SAA [**]	VSAA [**]
Sustained and unexplained cytopenia	ANC ≤ 0.5 x 10^9/l	ANC ≤ 0.2 x 10^9/l
At least bilineage morphologic myelodysplasia	Platelets < 20 x 10^9/l	Platelets < 20 x 10^9/l
Acquired clonal cytogenetic abnormalitiy in hematopoietic cells	Reticulocytes < 20 x 10^9/l	Reticulocytes < 20 x 10^9/l
Blasts: PB ≤ 2 %, BM ≤ 5%	< 30 % of normal cellularity in BM	< 30 % of normal cellularity in BM

[v]SAA [(very) severe aplastic anemia
ANC Absolute neutrophil count
PB Peripheral blood
BM Bone marrow
* Two of the first three criteria and criterion 4 are mandatory [10]
** all criteria should be fulfilled [7]

METHODS

TCR Vβ CDR3 repertoire analysis

Cryopreserved primary bone marrow and/or peripheral blood samples had been collected at diagnosis. Mononuclear cells (MNC) were isolated from PB and BM by Ficoll-Hypaque density centrifugation. RNA isolation was performed as described previously [17-18].

Following RNA isolation and cDNA synthesis, *TCRB* (Vβ-Cβ) PCR was applied [17-18]. Resulting PCR products were subjected to heteroduplex analysis to determine TCR Vβ CDR3 heterogeneity. The TCR Vβ CDR3 profiles were compared with those of the control patients.

Skewing of the TCR Vβ CDR3 repertoire was scored when an oligoclonal pattern in two or more lanes in the heteroduplex gel was found. Skewing in 2-5 lanes was defined as TCR score 1, whereas skewing in more than 5 lanes was defined as TCR score 2. In non-skewed cases polyclonal patterns were seen in all lanes, defined as TCR score 0.

In a proportion of cases consecutive follow-up samples were available for TCR Vβ CDR3 analysis to determine molecular response after IST.

Statistical analysis

Differences in TCR Vβ CDR3 repertoire score distribution between [v]SAA patients, MDS patients and healthy controls were calculated by χ^2 analysis. The difference between results of TCR Vβ CDR3 repertoire scores in peripheral blood and bone marrow were compared using Pearson correlation.

RESULTS AND DISCUSSION

In this study, a total of 84 cases were included. TCR Vβ CDR3 repertoire analysis was performed in 38 [v]SAA patients [n=17 SAA and n= 21 vSAA patients], 28 RCC patients and 18 controls. Of the RCC patients, 10/28 had a normal karyotype, in 16/28 cases cytogenetic abnormalities were found and in 2/28 cases the karyotype was unknown.

Oligoclonality (TCR scores 1 and 2) was found in 21/38 (55%) of the (v)SAA patients and in 17/28 (61%) of the RCC patients (χ^2 analysis, p=0.8). No difference in TCR score was found between the SAA and the vSAA patients (χ^2 analysis, p=0.28), nor between RCC-patients with a normal karyotype versus patients with cytogenetic abnormalities (χ^2 analysis, p=0.07). In two of the control cases, mild skewing (TCR score 1) was found: one was a healthy control and one was diagnosed with congenital bone marrow failure. A significant difference in TCR skewing pattern was found between (v)SAA + RCC patients as compared with the controls (χ^2 analysis, p=0.001), but not between RCC and (v)SAA patients (χ^2 analysis, p=0.8). Furthermore, no skewing of specific Vβ families was found when (v)SAA were compared with RCC patients (Figure 1).

Our retrospective TCR Vβ CDR3 repertoire study shows that 55% of (v)SAA had a skewed TCR pattern indicative of autoimmunity, which may reflect the proportion of patients who responded to immunosuppressive therapy in clinical pediatric (v)SAA studies (4).

So far no studies have been reported on TCR oligoclonality in pediatric MDS. Our results show that in 61% of the RCC patients autoimmunity apparently plays a role. This is comparable with findings in adult MDS patients, in which oligoclonality of the TCR Vβ CDR3 repertoire has been described in a high percentage of patients (14, 19-21). Moreover, in elderly MDS patients response to IST has been reported, which is indicative that an autoimmune attack may play a role in the pathogenesis of the disease (22-28). Recently, EWOG-MDS performed a pilot study in 31 children with hypoplastic RCC and normal karyotype in which a response rate of 71% at 6 months after initiation of IST was shown (29). This underscores our findings that auto-immunity may indeed play a role in subsets of pediatric MDS.

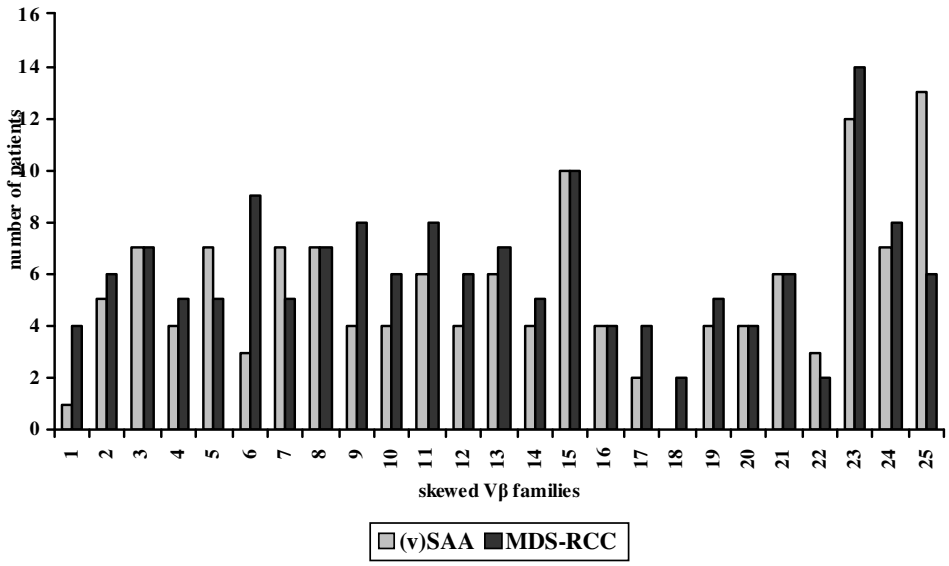


Figure 1: Skewed TCR Vβ CDR specificities in (v)SAA and MDS-RCC patients. TCR Vβ skewing frequency in peripheral blood and bone marrow samples of (v)SAA and MDS-RCC patients.

In two cases of pediatric (v)SAA consecutive follow-up PB samples were available for TCR Vβ CDR3 analysis to determine molecular response. In one clinically responding patient TCR Vβ CDR3 repertoire changed from TCR score 2 till 0 at 20 months after IST (cyclosporine, steroids and antithymocyte globulin) (Figure 2). The second patient, with an initial TCR score of 2, did not respond to IST and did not show a molecular response either. These findings illustrate that, as has been reported in adult patients TCR Vβ CDR3 analysis, this might be a valuable molecular response tool in children.

Of the 38 (v)SAA patients 22 received IST. Response data showed a response in 17 patients (8 CR and 9 PR) and no response in 4 patients. One patient died during treatment. Although the number of patients that received IST in the aplastic anemia patient group is considerable, it was not the intention of this retrospective study to investigate whether TCR Vβ CDR3 analysis is predictive for response, as the used IST regimens (dosages, timing, and compounds) and the moment of response assessment (4-7 months) were too variable for this analysis. Future prospective studies are necessary to investigate the value of this molecular tool for response measurement in pediatric (v)SAA and MDS-RCC patients.

Until now it is unkown which compartment (PB or BM) gives the best information on T-cell oligoclonality in autoimmune-mediated bone marrow failure. In this study, paired samples were available of 25 cases (Table 2), which showed a high level of correlation of TCR Vβ CDR3 repertoire scores between bone marrow and peripheral blood (Pearson correlation, $r^2 = 0.98$). Future studies are necessary to investigate which T-cell subtypes are responsible for this auto-immune phenomenon in respectively PB and BM.

In summary, in this retrospective study TCR Vβ CDR3 analysis was not discriminative between RCC and (v)SAA. It rather seems that there is overlap between (v)SAA and RCC patients, which possibly reflects a common pathogenesis. Prospective studies are necessary to investigate whether there is a role for this molecular tool in pediatric MDS to identify a subset of pediatric bone marrow failure/MDS patients that is associated with autoimmunity and therefore could be treated with IST up-front, and whether it can be employed for monitoring molecular response.

Table 2: Comparison of TCR Vβ CDR3 scores in paired bone marrow and peripheral blood samples.

	TCR score (PB)			
TCR score (BM)	**0**	**1**	**2**	**total**
0	11	2		13
1	3	1		4
2			8	8
total	14	3	8	25

BM = bone marrow, PB = peripheral blood, TCR = T-cell receptor, $r^2 = 0.98$

TCR score 0: no skewing: polyclonal pattern in all lanes.
TCR score 1: skewing: oligoclonality in 2-5 lanes.
TCR score 2: skewing: oligoclonality in more than 5 lanes.

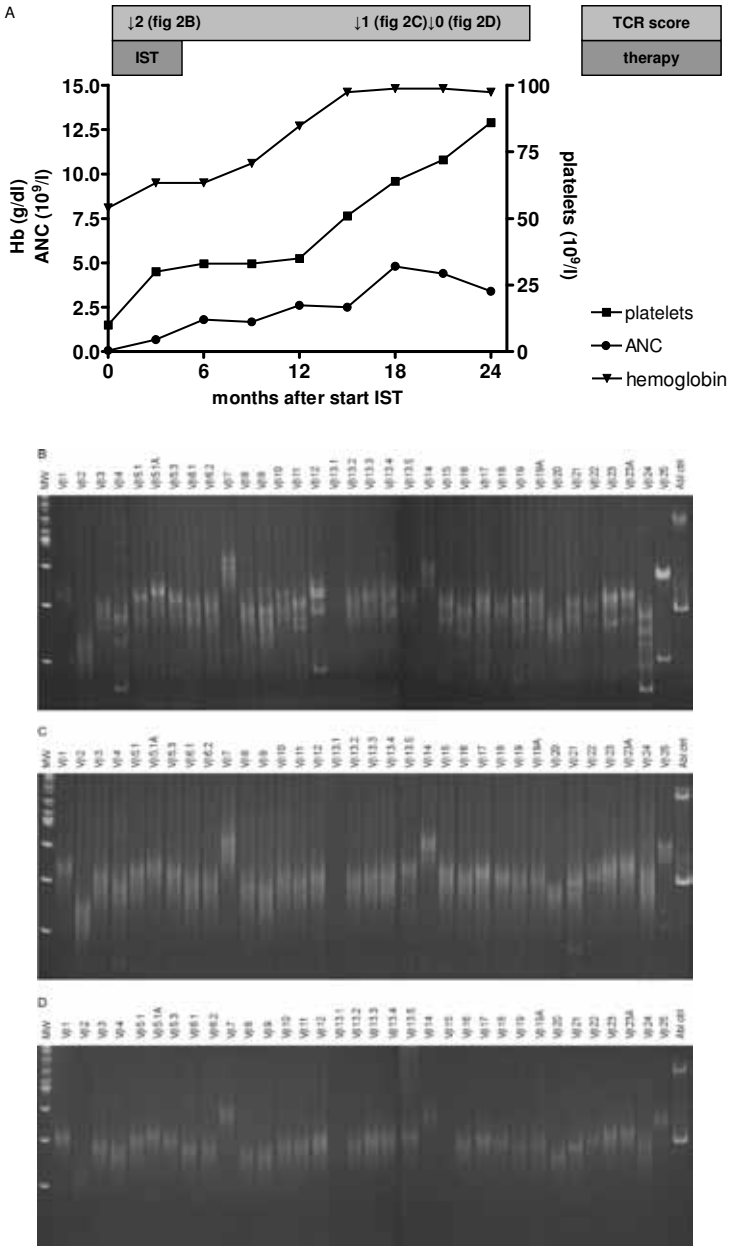
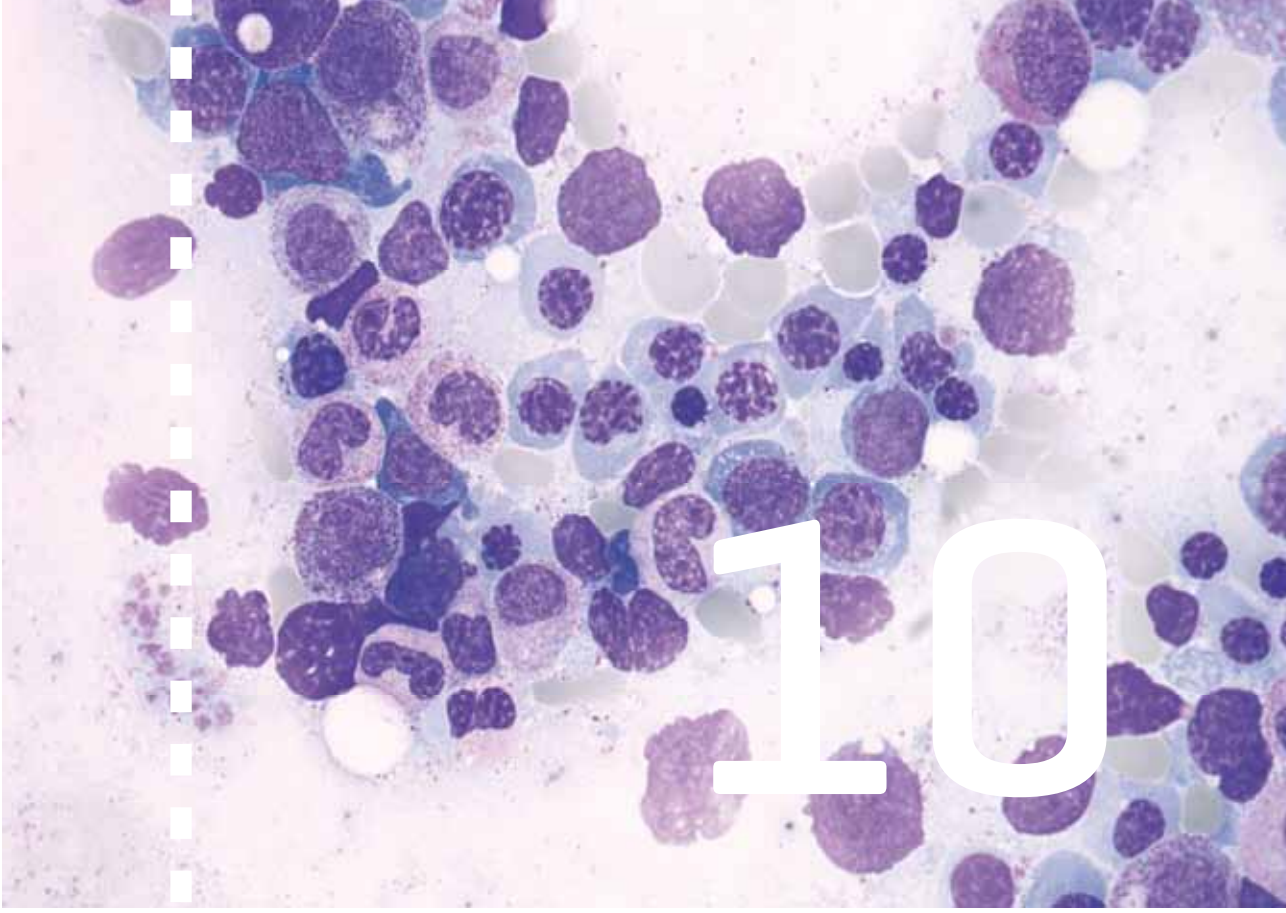


Figure 2: An individual patient with vSAA: haematological response and TCR Vβ CDR3 scores. **2A:** Hematological recovery after one course of IST (from month 0 until month 4) TCR scores at diagnosis, 18 months and 24 months after starting IST. **2B:** TCR Vβ repertoire analysis, a skewed pattern in Vβ4, Vβ7, Vβ11, Vβ12, Vβ24 and Vβ25, indicating a TCR score of 2, pattern before treatment. **2C:**TCR Vβ repertoire analysis, a skewed pattern in Vβ4, Vβ21 and Vβ25, indicating a TCR score of 1, 18 months after starting treatment. **2D:** TCR Vβ repertoire analysis, a polyclonal pattern, indicating a TCR score of 0, 24 months after starting treatment.

REFERENCES

1. Rosenfeld S, Follmann D, Nunez O, Young NS. Antithymocyte globulin and cyclosporine for severe aplastic anemia: association between hematologic response and long-term outcome. Jama. 2003 Mar 5;289(9):1130-5.
2. Young NS, Maciejewski J. The pathophysiology of acquired aplastic anemia. N Engl J Med. 1997 May 8;336(19):1365-72.
3. Brodsky R. Acquired severe aplastic anemia in children: is there a standard of care? Pediatr Blood Cancer. 2004 Dec;43(7):711-2.
4. Fuhrer M, Rampf U, Baumann I, Faldum A, Niemeyer C, Janka-Schaub G, et al. Immunosuppressive therapy for aplastic anemia in children: a more severe disease predicts better survival. Blood. 2005 Sep 15;106(6):2102-4.
5. Marsh JC. Results of immunosuppression in aplastic anaemia. Acta Haematol. 2000;103(1):26-32.
6. Bacigalupo A, Bruno B, Saracco P, Di Bona E, Locasciulli A, Locatelli F, et al. Antilymphocyte globulin, cyclosporine, prednisolone, and granulocyte colony-stimulating factor for severe aplastic anemia: an update of the GITMO/EBMT study on 100 patients. European Group for Blood and Marrow Transplantation (EBMT) Working Party on Severe Aplastic Anemia and the Gruppo Italiano Trapianti di Midolio Osseo (GITMO). Blood. 2000 Mar 15;95(6):1931-4.
7. Fuhrer M, Burdach S, Ebell W, Gadner H, Haas R, Harbott J, et al. Relapse and clonal disease in children with aplastic anemia (AA) after immunosuppressive therapy (IST): the SAA 94 experience. German/Austrian Pediatric Aplastic Anemia Working Group. Klin Padiatr. 1998 Jul-Aug;210(4):173-9.
8. Kojima S, Horibe K, Inaba J, Yoshimi A, Takahashi Y, Kudo K, et al. Long-term outcome of acquired aplastic anaemia in children: comparison between immunosuppressive therapy and bone marrow transplantation. Br J Haematol. 2000 Oct;111(1):321-8.
9. Kardos G, Baumann I, Passmore SJ, Locatelli F, Hasle H, Schultz KR, et al. Refractory anemia in childhood: a retrospective analysis of 67 patients with particular reference to monosomy 7. Blood. 2003 Sep 15;102(6):1997-2003.
10. Hasle H, Niemeyer CM, Chessells JM, Baumann I, Bennett JM, Kerndrup G, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia. 2003 Feb;17(2):277-82.
11. Kook H, Risitano AM, Zeng W, Wlodarski M, Lottemann C, Nakamura R, et al. Changes in T-cell receptor VB repertoire in aplastic anemia: effects of different immunosuppressive regimens. Blood. 2002 May 15;99(10):3668-75.
12. Risitano AM, Kook H, Zeng W, Chen G, Young NS, Maciejewski JP. Oligoclonal and polyclonal CD4 and CD8 lymphocytes in aplastic anemia and paroxysmal nocturnal hemoglobinuria measured by V beta CDR3 spectratyping and flow cytometry. Blood. 2002 Jul 1;100(1):178-83.
13. Risitano AM, Maciejewski JP, Green S, Plasilova M, Zeng W, Young NS. In-vivo dominant immune responses in aplastic anaemia: molecular tracking of putatively pathogenetic T-cell clones by TCR beta-CDR3 sequencing. Lancet. 2004 Jul 24-30;364(9431):355-64.
14. Wlodarski MW, Gondek LP, Nearman ZP, Plasilova M, Kalaycio M, Maciejewski JP. Molecular strategies for detection and quantitation of clonal cytotoxic T cell responses in aplastic anemia and myelodysplastic syndrome. Blood. 2006 Apr 13.
15. Camitta BM, Thomas ED, Nathan DG, Santos G, Gordon-Smith EC, Gale RP, et al. Severe aplastic anemia: a prospective study of the effect of early marrow transplantation on acute mortality. Blood. 1976 Jul;48(1):63-70.
16. Cantu Rajnoldi A, Fenu S, Kerndrup G, van Wering ER, Niemeyer CM, Baumann I. Evaluation of dysplastic features in myelodysplastic syndromes: experience from the morphology group of the European Working Group of MDS in Childhood (EWOG-MDS). Ann Hematol. 2005 Jul;84(7):429-33.
17. Langerak AW, Szczepanski T, van der Burg M, Wolvers-Tettero IL, van Dongen JJ. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. Leukemia. 1997 Dec;11(12):2192-9.
18. Langerak AW, van Den Beemd R, Wolvers-Tettero IL, Boor PP, van Lochem EG, Hooijkaas H, et al. Molecular and flow cytometric analysis of the Vbeta repertoire for clonality assessment in mature TCRalphabeta T-cell proliferations. Blood. 2001 Jul 1;98(1):165-73.
19. Epperson DE, Nakamura R, Saunthararajah Y, Melenhorst J, Barrett AJ. Oligoclonal T cell expansion in myelodysplastic syndrome: evidence for an autoimmune process. Leuk Res. 2001 Dec;25(12):1075-83.
20. Plasilova M, Risitano A, Maciejewski JP. Application of the molecular analysis of the T-cell receptor repertoire in the study of immune-mediated hematologic diseases. Hematology. 2003 Jun;8(3):173-81.
21. Sloand EM, Mainwaring L, Fuhrer M, Ramkissoon S, Risitano AM, Keyvanafar K, et al. Preferential suppression of trisomy 8 compared with normal hematopoietic cell growth by autologous lymphocytes in patients with trisomy 8 myelodysplastic syndrome. Blood. 2005 Aug 1;106(3):841-51.
22. Asano Y, Maeda M, Uchida N, Yokoyama T, Osaki K, Shimoda K, et al. Immunosuppressive therapy for patients with refractory anemia. Ann Hematol. 2001 Nov;80(11):634-8.
23. Biesma DH, van den Tweel JG, Verdonck LF. Immunosuppressive therapy for hypoplastic myelodysplastic syndrome. Cancer. 1997 Apr 15;79(8):1548-51.

24. Jonasova A, Neuwirtova R, Cermak J, Vozobulova V, Mocikova K, Siskova M, et al. Cyclosporin A therapy in hypoplastic MDS patients and certain refractory anaemias without hypoplastic bone marrow. Br J Haematol. 1998 Feb;100(2):304-9.
25. Molldrem JJ, Leifer E, Bahceci E, Saunthararajah Y, Rivera M, Dunbar C, et al. Antithymocyte globulin for treatment of the bone marrow failure associated with myelodysplastic syndromes. Ann Intern Med. 2002 Aug 6;137(3):156-63.
26. Stadler M, Germing U, Kliche KO, Josten KM, Kuse R, Hofmann WK, et al. A prospective, randomised, phase II study of horse antithymocyte globulin vs rabbit antithymocyte globulin as immune-modulating therapy in patients with low-risk myelodysplastic syndromes. Leukemia. 2004 Mar;18(3):460-5.
27. Tamayose K, Sugimoto K, Ando M, Oshimi K. Disappearance of chromosomal abnormalities and recovery of hematopoiesis after immunosuppressive therapy for hypoplastic refractory anemia with excess of blasts. Blood. 2001 Apr 15;97(8):2524.
28. Yamada T, Tsurumi H, Kasahara S, Hara T, Sawada M, Moriwaki H. Immunosuppressive therapy for myelodysplastic syndrome: efficacy of methylprednisolone pulse therapy with or without cyclosporin A. J Cancer Res Clin Oncol. 2003 Aug;129(8):485-91.
29. Yoshimi A, Baumann I, Fuhrer M, Bergstrasser E, Gobel U, Sykora KW, et al. Immunosuppressive therapy with anti-thymocyte globulin and cyclosporine A in selected children with hypoplastic refractory cytopenia. Haematologica. 2007 Mar;92(3):397-400.



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SUMMARY DISCUSSION AND FUTURE PERSPECTIVES

Illustration: MDS, dyserythropoiesis (BM)

Juvenile myelomonocytic leukemia and myelodysplastic syndrome are rare diseases in childhood that belong to the myeloproliferative/myelodysplastic disorders in the WHO classification. Until now, stem cell transplantation is the only curative option for both diseases. This procedure harbours the risk of serious toxicity and mortality, not only during the procedure, but also in later life. Hence, there is a need for unravelling the pathogenesis of pediatric JMML and MDS to identify potential drugable targets. In this thesis we studied several aspects of the biology of JMML and MDS.

PART I: JUVENILE MYELOMONOCYTIC LEUKEMIA

Summary

In the first part of this thesis we studied different aspects of the molecular backgrounds of JMML. In chapter 1 we give an overview of the known involved molecular aberrations and we describe the current classification, which can be used in the diagnostic process of an individual case of JMML. JMML is a disease in which the activation of the RAS-RAF-MEK-ERK-pathway plays an essential role. *PTPN11* mutations are found in 35%, *RAS* mutations in 25%, and *NF1* in 11% of the JMML patients. More recently, the clinical observation of a specific phenotype in a subgroup of JMML patients resulted in identifying *c-CBL* mutations as pathogenetic drivers in 17%. So, currently in about 85% of the JMML patients a molecular aberration is identified.

In chapter 2 we investigated whether mutations in *BRAF,* another gene involved in the RAS-RAF-MEK-ERK signal transduction pathway, could be identified in 65 JMML patients. However, we could not identify any *BRAF* mutations in any of the the JMML patients, indicating that this gene is not an important involved gene in JMML.

In chapter 3 we report on *FLT3* studies in JMML. In acute myeloid leukemia (AML) M4/M5 (the myelomonocytic types of AML) and in infant acute lymphoid leukemia this gene is highly expressed due to internal tandem duplications (*ITDs*) and mutations or wild-type overexpression by ligand overexpression. In JMML, we found no evidence for *FLT3* overexpression, nor did we detect *ITD* or mutations in the *FLT3* gene nor overexpression of the *FLT3* ligand. We therefore conclude, that *FLT3* abnormalities are not important in the pathogenesis of JMML and that there is no rationale to assume that JMML patients might benefit from FLT3 inhibitors.

Chapter 4 describes *WT1* studies in JMML. We found *WT1* overexpression in JMML patients as compared to healthy controls, but to a lower extent as in pediatric AML. In 4% of JMML patients we identified a mutation in the *WT1* gene, which was not related to overexpression. In addition, in contrast to childhood AML, the occurrence of a common polymorphism (rs16754) was not related to outcome. Unfortunately, we conclude that *WT1* is not important in the pathogenesis of JMML.

Until now stem cell transplantation is the only curative treatment option in JMML. Currently, families are small with mostly 1-2 siblings available all with only 25% probability of being a suitable HLA matched donor. When a young mother is pregnant at the time of the diagnosis of a JMML patient, cord blood transplantation can be considered in case of HLA identity. The contribution of graft-versus-leukemia was considered to be important, especially in this particular disease (1, 2). As it was not known whether HLA identical cord blood sibling transplantation was a reasonable strategy, we studied the feasibility of this stem cell source in children with JMML. In the small international series available

we showed that this stem cell source can be used as a reliable alternative for matched related or unrelated bone marrow.

JMML: DISCUSSION AND FUTURE PERSPECTIVES

Diagnostic challenges

JMML is a rare disease in childhood with a wide spectrum of clinical symptoms (3). It is a rapidly fatal disorder for most children if left untreated and therefore early recognition of the disease is necessary. To achieve this, it is important that pediatricians as well as pediatric oncologists improve their knowledge about the clinical symptoms of JMML. In case of a clinical picture of JMML it is a diagnostic challenge to investigate which molecular aberration is the driving aberration. Until now, only few studies have been published about the prognostic role of the different RAS pathway mutations. Small studies have suggested a poor prognostic implication of somatic *PTPN11* mutations (4, 5), although this has not been confirmed in the EWOG-MDS cohort so far (6).

The other value of molecular diagnostics is to identify rare JMML patients with an underlying syndrome, as Noonan syndrome and in some *c-CBL* mutated cases, which have been described to resolve spontaneously (7-12). Future studies with large numbers in international registries are necessary for identifying those patients who can be carefully followed without stem cell transplantation (13).

We found that the 15% of the JMML cases without known *RAS* pathway mutations were not regulated by mutations or overexpression of other genes, such as *WT1*, *FLT3* and *BRAF*. Recently, a genome wide analysis of JMML patients showed that patients who displayed an AML-type signature had a worse prognosis compared to patients without an AML-like expression profile (10-years EFS of 6% vs 63%). The expression profile was not correlated to a specific mutation status and the earlier mentioned lack of prognostic significance of specific mutations suggest that other factors are responsible for the disease, which have to be unravelled in the future (14).

The epigenetic studies, which are currently performed, may give new information for the use of new therapeutic options, such as the use of demethylating agents. In addition, more advanced research tools, such as high throughput sequencing, which has shown to be successful in acute myeloid leukemia and myelodysplastic syndrome, may point to novel involved and deregulated genes and signal transduction pathways (15, 16). Moreover, the RAS-RAF-MEK-ERK pathway may be activated in other way than by mutations.

Therapeutic challenges

Until now, stem cell transplantation is the only curative option for JMML. This carries a high risk of treatment related morbidity and mortality and does not prevent relapses in about one third of the cases. The event free survival at 5 years after transplantation is about 50%, which has not improved in the last decade. Therefore new therapeutic approaches are urgently needed.

In order to improve outcome it is a challenge to design uniform conditioning regimens and to select the most suitable donors, leading to a decrease in both treatment related toxicity and relapse. We showed that umbilical cord blood can be used as an alternative donor source, even in case of immunity naïve HLA-identical siblings.

Also optimising conditioning regimens should be a challenge, including patient tailored dosing regimens, such as bulsulphan in which intravenous dosing with therapeutic drug monitoring seems to be preferable over oral busulphan in children (17). Alternatively, treosulphan-based myeloablative conditioning provides also a high rate of primary engraftment and favourable acute toxicity profile in children (18). As JMML is a rare disease, all attempts to improve the treatment should be performed in international collaborative studies and in centers for pediatric stem cell transplantation where substantial numbers of patients are treated. This will increase survival and hopefully decrease serious stem cell related side effects.

As in 85% of the JMML patients RAS-pathway mutations are involved, targeting the RAS-RAF-MEK-ERK pathway would be the most logical therapeutic approach as an alternative for HSCT (19). A Phase II study with a farnesyltranseferase inhibitor (tipifarnib) showed a partial clinical response prior to transplantation, but there was no effect on the EFS (13, 20).

Other RAS-RAF-MEK-ERK inhibitors, like Sorafenib, have been developed and analyzed in leukemia and solid tumors. The efficacy has not been evaluated in JMML so far. Although many of these inhibitors are promising, one of the problems is that RAS signaling is also involved in normal cells, so inhibiting of RAS may not be as specific as suggested. Moreover, the effect of the RAS pathway inhibitors may be bypassed by mutations in other signaling pathways, like the P13K/PTEN/Akt/mTOR pathways (19). A novel promising technique to search for specific RAS pathway inhibitors and other potential effective agents in JMML could be the in silico and in vitro use of drug libraries, in which large series of compounds can be tested for their ability to modify the target, in combination with biomic profiles (21).

In our study we have analysed potential new therapeutic targets in JMML. We did not identify mutations in the *FLT3* and *BRAF* genes, which suggests that it is unlikely that JMML patients will benefit from *FLT3* inhibitors or *BRAF* inhibitors.

As *WT1* is one of the genes, which is indirectly regulating the RAS-RAF-MEK-ERK pathway, we anticipated that this would have been an interesting therapeutic target, but we couldn't provide that evidence, which is unfortunate as in AML and solid tumours *WT1* is used as a new therapeutic target for immunotherapy (22, 23).

Hence, for the future a promising mechanism, which regulates the activity of signalling pathways, is hypermethylation in JMML. This hypermethylation was associated with an aggressive biologic variant of JMML and was found to be an important predictor of outcome (24). Therefore it is reasonable that demethylating agents can be used, which was illustrated by a case report with a complete morphological and molecular remission with demethylating agents (25). These findings are now being implemented in an international EWOG-MDS/ITCC phase I/II trial with the demethylating agent azacitidine in JMML, which has started in 2012.

In conclusion, in 15% of JMML patients no molecular aberration is found, and future diagnostic challenges will be necessary to unravel the molecular characteristics of this group. In addition, studies are needed to identify those patients who might benefit from a watch and wait strategy. Further studies in JMML will result in new therapeutic options and hopefully in the future provide alternatives for aggressive regimens such as stem cell transplantation in these young children. In a rare disease like JMML it is of great importance that for the evaluation of the effect of new agents like RAS pathway inhibitors

or of specific transplantation regimens, studies are being performed in international collaboration.

PART II: MYELODYSPLASTIC SYNDROME

Summary

Until now only scarce information is available about the pathogenesis of childhood MDS. The second part of this thesis describes the studies we performed to get insight in the pathogenesis of childhood MDS. The results make clear that the biology of childhood MDS is different from that in adult MDS.

We studied the occurrence of molecular aberrations in 107 childhood MDS cases (chapter 6). The incidence of type I and II aberrations appeared to be lower than in adult MDS. Especially, genes involved in histone modification and DNA methylation are less frequently mutated in children as compared to adults. This underscores that aging influences the occurrence of mutations in these genes.

Using array-CGH we found in a relative large subgroup of pediatric MDS patients with chromosome 6 aberrations a common breakpoint in the *IER3* gene. This gene is considered to be involved in the pathogenesis of adult MDS. We found that *IER3* expression is low in childhood MDS and that this low expression was associated with a poor outcome. Epigenetic studies showed that this downregulation was not regulated by hypermethylation of the *IER3* promoter region (chapter 7).

A novel molecular pathogenetic factor in pediatric MDS seems to be the occurrence of mutations in the mitochondrial DNA (mtDNA). We found that these aberrations occur in 68% of sporadic childhood MDS cases as germline events or as somatic aberrations (chapter 8). This frequency is higher as compared to the frequency in adult MDS patients, which is surprising, as it previously has been suggested that the incidence of mtDNA mutations is influenced by aging. We hypothesize that in children, mtDNA mutations induce vulnerability for second hits in the hematopoietic stem cell. Whether this is a more general mechanism of predisposition of pediatric cancer needs to be determined.

In chapter 9 the auto-immune background in subsets of MDS patients is described. We investigated the occurrence of T-Cell receptor Vβ oligoclonality in childhood MDS and severe aplastic anemia (SAA). The frequency of TCR Vβ repertoire skewing in both MDS and SAA is around 60%, indicating that the analysis of the TCR repertoire skewing can not be used to differentiate between both diseases. However, it underscores the hypothesis that in a subset of children the MDS is caused by auto-immune deregulation, which may benefit from immunosuppressive therapy, instead of upfront SCT.

MDS: DISCUSSION AND FUTURE PERSPECTIVES

Diagnostics challenges

Currently, the diagnosis of myelodysplastic syndrome is based on histological aberrations and discrepancies between histology and morphology are frequently described (26, 27). Typical karyotyping findings are associated with MDS, such as monosomy 7.

Of MDS-RCC patients 60-80% have a hypocellular bone marrow, which makes the differentiation between severe aplastic anemia and refractory cytopenia difficult, especially in case of normal karyotype (7, 28). The discrimination between these two

diseases is important as until recently, MDS-RCC is treated with stem cell transplantation, whereas severe aplastic anemia is primary treated with immunosuppressive therapy. Our study using TCR Vβ analyses showed that auto-immunity is also involved in a subset of MDS-RCC patients. This finding resulted in an ongoing prospective study, in which the clinical relevance of TCR Vβ oligoclonality, the occurrence of glycosylphosphatidylinositol (GPI) anchor deficient clones (PNH clones), T-cell subtypes and immunophenotype are analysed. This study may identify the MDS-RCC patients who may be selected to benefit from immunosuppressive therapy and for whom stem cell transplantation can be omitted.

We showed that the frequency of recurrent molecular aberrations in childhood MDS is lower as compared to adults, which was especially obvious for the aberrations in genes involved in DNA methylation and histone modification (29-31). Other recent studies reported that also, mutations in the RNA splicing genes are less frequent in childhood MDS (15, 32-34). Altogether this indicates that childhood MDS may be a different biological entity than adult MDS.

New high throughput techniques like whole genome sequencing and RNA sequencing, which have shown novel molecular aberrations in acute myeloid leukemia and adult MDS, may be helpful for further unravelling the pathogenesis of childhood MDS (16, 34). Using such techniques, mutations in the *IDH1*, *IDH2* gene and the *DNMT3A* gene, involved in DNA methylation and histone modification, were recently identified in adult MDS.

Interestingly, in this thesis we found that 68% of the pediatric MDS patients carried a mtDNA mutation. This frequency is higher than in adult MDS cases, which is striking, especially when taking into account that the frequency of mtDNA increases with aging (35). We identified somatic mtDNA mutations, which we suggest to act as a first hit in the bone marrow, which makes the hematopoietic stem cell more vulnerable for subsequent aberrations. We also identified germline mtDNA mutations, which probably may lead to a general increased risk for additional aberrations, not only for the hematopoietic stem cell, but for all cells, resulting in a general increased risk for the development of cancer. Recently, it was shown that nuclear microRNAs (miRNAs) translocates into the mitochondrial genome and regulates the mitochondrial protein synthesis and thereby the function of the oxidative phosphorylation (36). MiRNAs are regulators of human hematopoietic stem cells and their deregulation contributes to the development of haematological malignancies. In pediatric acute myeloid leukemia the expression of miRNAs is different in clinically relevant cytogenetic subgroups which suggests a role in the underlying biology of the leukemia (37). There is also evidence that deregulation of miRNAs contribute to the pathogenesis of MDS (38).

In addition, in adults a decreased expression of mitochondrial encoded genes in adults with acute myeloid leukemia and dysplasia related changes was found. Further studies will be necessary to address the role of the mtDNA mutations, expression levels of mitochondrial encoded genes, miRNAs and the oxidative phosphorylation in the pathogenesis of childhood MDS, but also in other types of childhood cancer.

Therapeutic challenges in pediatric MDS

As for JMML, the development of better transplant conditioning regimens for MDS patients is a challenge. Until now a conditioning regimen with busulphan, cyclophosfamide and melphalan is used. Therapeutic drug monitoring of intravenously busulphan may make

the use of melphalan superfluous, thereby decreasing the direct and late toxicity, without increasing the relapse rate and graft failure rate (17). An alternative approach might be a treosulphan based conditioning regimen. For studies on the best conditioning regimen international collaborations remain necessary. There is some evidence that subsets of MDS patients (MDS-RCC with a matched donor) can be treated with reduced intensity conditioning, which reduces toxicity, but long term follow up is necessary (39).

The development of alternatives for stem cell transplantation as treatment of MDS would be very valuable. The involvement of autoimmunity in a subset of patients provides opportunities for immunosuppressive therapy such as anti-thymocyte globulin (ATG) and steroids. Currently, the EWOG-MDS-group runs an international study on the effectiveness of immunosuppressive therapy with cyclosporine A and anti-thymocyte globulin (ATG) for RCC patients with hypocellular bone marrow with a normal karyotype or trisomy 8, for whom no suitable HLA matched sibling donor is available. A randomized study in adults with SAA showed that the use of rabbit ATG resulted in inferior outcome compared to horse ATG (40). Unpublished observational data of the EWOG-MDS group did not reveal differences in clinical outcome between administration of horse and rabbit ATG (unpublished data).

In a subset of adult MDS patients autoimmunity is reflected by the presence of PNH clones, i.e. hematopoietic cells that are deficient in glucophosphatidylinositol anchored proteins. The presence of PNH clones makes hematopoietic cells susceptible to autoimmunity. The frequency of PNH clones and the relevance of the clone size in children with MDS is currently investigated in a prospective EWOG-MDS study. Recently, it was demonstrated that eculizumab, a complement inactivator, has a therapeutic effect in adult patients with paroxysmal nocturnal hemoglobinuria disease. It is unknown whether it might be a future therapeutic option in childhood MDS with small PNH clones.

Besides genetic aberrations and auto-immunity, epigenetics are suspected to be involved in the development of MDS (41). Epigenetics refer to a number of biochemical modifications of chromatin that have a role in genomic regulation and especially gene expression control, without altering the primar sequence of the DNA. The DNA methyltransferase inhibitor azacitidine gives a significantly better survival in adult MDS patients compared to the conventional treatment (42). This has prompted the design of a EWOG-MDS/ITCC phase I/II study with azacitidine in children with MDS. Pharmacokinetics and dynamics of azacitidine will be investigated, together with the analyses of the methylation status of specific genes involved in the pathogenesis of MDS.

In this thesis we showed that mtDNA mutations are involved in the pathogenesis of childhood MDS, which apart from the importance of understanding the biology may have consequences for donor selection. Therefore we recommend analysis of the bone marrow of potential matched related donors for histology, cytogenetic analysis and analysis of mtDNA aberrations to prevent that MDS patients will be transplanted with stem cells which carry similar mtDNA mutations or another molecular aberration as first hit necessary for the development of MDS.

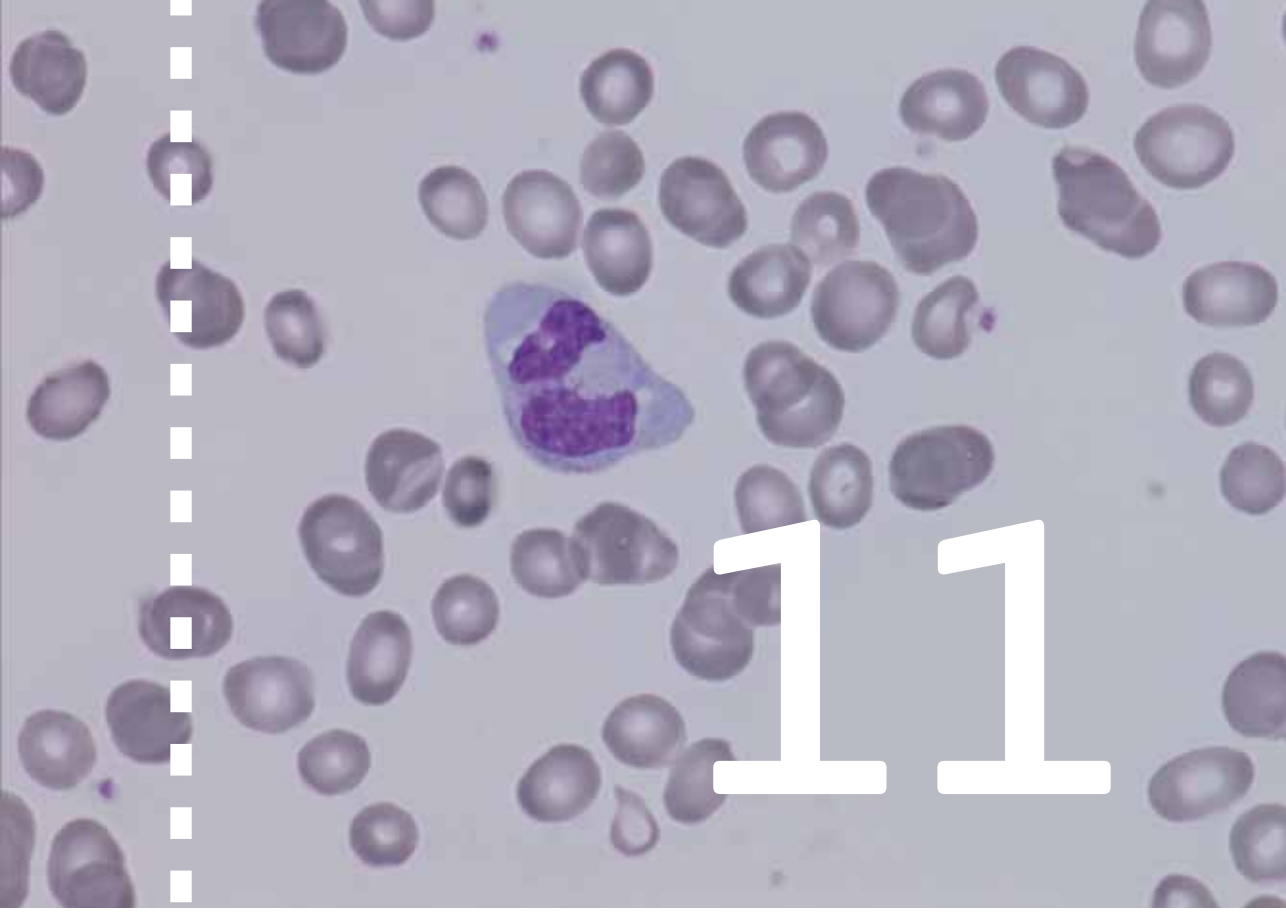
In conclusion, in this thesis we showed that the frequency of the known type I/II aberrations is lower in childhood MDS as compared to adult MDS, especially in genes involved in epigenetic regulation. This suggests that alternative mechanisms are responsible for the development of MDS in children. We have shown that one of these mechanisms might be the frequent occurrence of mtDNA mutations. Furthermore, in a

subset of MDS-RCC patients an auto-immune attack is involved. Further research will result in the identification of patients which may benefit from specific treatment options, to decrease toxicity and increase the efficacy of the current treatment regimens.

REFERENCES

1. Fuchs O. [EVI1 and its role in myelodysplastic syndrome, myeloid leukemia and other malignant diseases]. Cas Lek Cesk. 2006;145(8):619-24. Epub 2006/09/26. EVI1 a jeho uloha u myelodysplastickych syndromu, myeloidni leukemie a dalsich malignich onemocneni.
2. Smith FO, King R, Nelson G, Wagner JE, Robertson KA, Sanders JE, et al. Unrelated donor bone marrow transplantation for children with juvenile myelomonocytic leukaemia. Br J Haematol. 2002;116(3):716-24.
3. Niemeyer CM, Arico M, Basso G, Biondi A, Cantu Rajnoldi A, Creutzig U, et al. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). Blood. 1997;89(10):3534-43.
4. Hollink IH, Zwaan CM, Zimmermann M, Arentsen-Peters TC, Pieters R, Cloos J, et al. Favorable prognostic impact of NPM1 gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML. Leukemia. 2009;23(2):262-70. Epub 2008/11/21.
5. Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, Zimmermann M, Peeters JK, Valk PJ, et al. Characterization of CEBPA mutations and promoter hypermethylation in pediatric acute myeloid leukemia. Haematologica. 2011;96(3):384-92. Epub 2010/12/08.
6. Locatelli F, Nollke P, Zecca M, Korthof E, Lanino E, Peters C, et al. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): results of the EWOG-MDS/EBMT trial. Blood. 2005;105(1):410-9.
7. EWOG-MDS. Clinical Trial Protocol EWOG-MDS 2006. Prospective non-randomized multi-center study for epidemiology and characterization of Myelodysplastic Syndromes (MDS) and Juvenile Myelomonocytic Leukemia (JMML) in childhood. 2007.
8. Bader-Meunier B, Tchernia G, Mielot F, Fontaine JL, Thomas C, Lyonnet S, et al. Occurrence of myeloproliferative disorder in patients with Noonan syndrome. The Journal of pediatrics. 1997;130(6):885-9.
9. Fukuda M, Horibe K, Miyajima Y, Matsumoto K, Nagashima M. Spontaneous remission of juvenile chronic myelomonocytic leukemia in an infant with Noonan syndrome. J Pediatr Hematol Oncol. 1997;19(2):177-9.
10. Silvio F, Carlo L, Elena B, Nicoletta B, Daniela F, Roberto M. Transient abnormal myelopoiesis in Noonan syndrome. J Pediatr Hematol Oncol. 2002;24(9):763-4.
11. Niemeyer CM, Kang MW, Shin DH, Furlan I, Erlacher M, Bunin NJ, et al. Germline CBL mutations cause developmental abnormalities and predispose to juvenile myelomonocytic leukemia. Nat Genet. 2010;42(9):794-800. Epub 2010/08/10.
12. Loh ML, Sakai DS, Flotho C, Kang M, Fliegauf M, Archambeault S, et al. Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. Blood. 2009;114(9):1859-63. Epub 2009/07/03.
13. Jadersten M, Saft L, Pellagatti A, Gohring G, Wainscoat JS, Boultwood J, et al. Clonal heterogeneity in the 5q- syndrome: p53 expressing progenitors prevail during lenalidomide treatment and expand at disease progression. Haematologica. 2009;94(12):1762-6. Epub 2009/10/03.
14. Bresolin S, Zecca M, Flotho C, Trentin L, Zangrando A, Sainati L, et al. Gene expression-based classification as an independent predictor of clinical outcome in juvenile myelomonocytic leukemia. J Clin Oncol. 2010;28(11):1919-27. Epub 2010/03/17.
15. Damm F, Bunke T, Thol F, Markus B, Wagner K, Gohring G, et al. Prognostic implications and molecular associations of NADH dehydrogenase subunit 4 (ND4) mutations in acute myeloid leukemia. Leukemia. 2011.
16. Andersen MT, Andersen MK, Christiansen DH, Pedersen-Bjergaard J. NPM1 mutations in therapy-related acute myeloid leukemia with uncharacteristic features. Leukemia. 2008;22(5):951-5. Epub 2008/02/15.
17. Bartelink IH, Bredius RG, Ververs TT, Raphael MF, van Kesteren C, Bierings M, et al. Once-daily intravenous busulfan with therapeutic drug monitoring compared to conventional oral busulfan improves survival and engraftment in children undergoing allogeneic stem cell transplantation. Biol Blood Marrow Transplant. 2008;14(1):88-98. Epub 2007/12/27.
18. Cutting R, Mirelman A, Vora A. Treosulphan as an alternative to busulphan for myeloablative conditioning in paediatric allogeneic transplantation. Br J Haematol. 2008;143(5):748-51. Epub 2008/11/28.
19. Flotho C, Kratz C, Niemeyer CM. Targeting RAS signaling pathways in juvenile myelomonocytic leukemia. Curr Drug Targets. 2007;8(6):715-25. Epub 2007/06/23.
20. Pardanani A, Patnaik MM, Lasho TL, Mai M, Knudson RA, Finke C, et al. Recurrent IDH mutations in high-risk myelodysplastic syndrome or acute myeloid leukemia with isolated del(5q). Leukemia. 2010;24(7):1370-2. Epub 2010/05/21.

21. Labarbera DV, Reid BG, Yoo BH. The multicellular tumor spheroid model for high-throughput cancer drug discovery. Expert Opin Drug Discov. 2012. Epub 2012/07/14.
22. Gao L, Bellantuono I, Elsasser A, Marley SB, Gordon MY, Goldman JM, et al. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. Blood. 2000;95(7):2198-203. Epub 2000/03/25.
23. Weber G, Karbach J, Kuci S, Kreyenberg H, Willasch A, Koscielniak E, et al. WT1 peptide-specific T cells generated from peripheral blood of healthy donors: possible implications for adoptive immunotherapy after allogeneic stem cell transplantation. Leukemia. 2009;23(9):1634-42. Epub 2009/04/10.
24. Olk-Batz C, Poetsch AR, Nollke P, Claus R, Zucknick M, Sandrock I, et al. Aberrant DNA methylation characterizes juvenile myelomonocytic leukemia with poor outcome. Blood. 2011;117(18):4871-80. Epub 2011/03/17.
25. Furlan I, Batz C, Flotho C, Mohr B, Lubbert M, Suttorp M, et al. Intriguing response to azacitidine in a patient with juvenile myelomonocytic leukemia and monosomy 7. Blood. 2009;113(12):2867-8. Epub 2009/03/21.
26. Cantu Rajnoldi A, Fenu S, Kerndrup G, van Wering ER, Niemeyer CM, Baumann I. Evaluation of dysplastic features in myelodysplastic syndromes: experience from the morphology group of the European Working Group of MDS in Childhood (EWOG-MDS). Annals of hematology. 2005;84(7):429-33.
27. Baumann I, Fuhrer M, Behrendt S, Campr V, Csomor J, Furlan I, et al. Morphological differentiation of severe aplastic anaemia from hypocellular refractory cytopenia of childhood: reproducibility of histopathological diagnostic criteria. Histopathology. 2012;61(1):10-7. Epub 2012/03/31.
28. Kardos G, Baumann I, Passmore SJ, Locatelli F, Hasle H, Schultz KR, et al. Refractory anemia in childhood: a retrospective analysis of 67 patients with particular reference to monosomy 7. Blood. 2003;102(6):1997-2003.
29. Wang GG, Cai L, Pasillas MP, Kamps MP. NUP98-NSD1 links H3K36 methylation to Hox-A gene activation and leukaemogenesis. Nat Cell Biol. 2007;9(7):804-12. Epub 2007/06/26.
30. Bojesen SE, Ammerpohl O, Weinhausl A, Haas OA, Mettal H, Bohle RM, et al. Characterisation of the GRAF gene promoter and its methylation in patients with acute myeloid leukaemia and myelodysplastic syndrome. Br J Cancer. 2006;94(2):323-32. Epub 2006/01/13.
31. Bains A, Luthra R, Medeiros LJ, Zuo Z. FLT3 and NPM1 mutations in myelodysplastic syndromes: Frequency and potential value for predicting progression to acute myeloid leukemia. Am J Clin Pathol. 2011;135(1):62-9. Epub 2010/12/22.
32. Hirabayashi S, Flotho C, Moetter J, Heuser M, Hasle H, Gruhn B, et al. Spliceosomal gene aberrations are rare, coexist with oncogenic mutations, and are unlikely to exert a driver effect in childhood MDS and JMML. Blood. 2012;119(11):e96-9. Epub 2012/01/13.
33. Klauke K, de Haan G. Polycomb group proteins in hematopoietic stem cell aging and malignancies. International journal of hematology. 2011;94(1):11-23. Epub 2011/04/28.
34. Haferlach C, Bacher U, Haferlach T, Dicker F, Alpermann T, Kern W, et al. The inv(3)(q21q26)/t(3;3)(q21;q26) is frequently accompanied by alterations of the RUNX1, KRAS and NRAS and NF1 genes and mediates adverse prognosis both in MDS and in AML: a study in 39 cases of MDS or AML. Leukemia. 2011;25(5):874-7. Epub 2011/02/02.
35. Chinnery PF, Samuels DC, Elson J, Turnbull DM. Accumulation of mitochondrial DNA mutations in ageing, cancer, and mitochondrial disease: is there a common mechanism? Lancet. 2002;360(9342):1323-5. Epub 2002/11/05.
36. Das S, Ferlito M, Kent OA, Fox-Talbot K, Wang R, Liu D, et al. Nuclear miRNA Regulates the Mitochondrial Genome in the Heart. Circ Res. 2012;110(12):1596-603. Epub 2012/04/21.
37. Danen-van Oorschot AA, Kuipers JE, Arentsen-Peters S, Schotte D, de Haas V, Trka J, et al. Differentially expressed miRNAs in cytogenetic and molecular subtypes of pediatric acute myeloid leukemia. Pediatr Blood Cancer. 2012;58(5):715-21. Epub 2011/08/06.
38. Dicker F, Haferlach C, Sundermann J, Wendland N, Weiss T, Kern W, et al. Mutation analysis for RUNX1, MLL-PTD, FLT3-ITD, NPM1 and NRAS in 269 patients with MDS or secondary AML. Leukemia. 2010;24(8):1528-32. Epub 2010/06/04.
39. Strahm B, Locatelli F, Bader P, Ehlert K, Kremens B, Zintl F, et al. Reduced intensity conditioning in unrelated donor transplantation for refractory cytopenia in childhood. Bone Marrow Transplant. 2007;40(4):329-33. Epub 2007/06/26.
40. Scheinberg P, Nunez O, Weinstein B, Biancotto A, Wu CO, Young NS. Horse versus rabbit antithymocyte globulin in acquired aplastic anemia. N Engl J Med. 2011;365(5):430-8. Epub 2011/08/05.
41. Fuchs O, Provaznikova D, Kocova M, Kostecka A, Cvekova P, Neuwirtova R, et al. CEBPA polymorphisms and mutations in patients with acute myeloid leukemia, myelodysplastic syndrome, multiple myeloma and non-Hodgkin's lymphoma. Blood cells, molecules & diseases. 2008;40(3):401-5. Epub 2008/01/10.
42. Fenaux P, Mufti G, Santini V, al. e. Azacitidine (AZA) treatment prolongs overall survival in higher-risk MDS patients compared with conventional care regimens (CCR): results of the AZA-001 phase III study. Blood. 2007;110(250a):abstract 817.



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NEDERLANDSE SAMENVATTING

MOLECULAIRE AFWIJKINGEN BIJ JUVENIELE MYELOMONOCYTAIRE LEUKEMIE (JMML) EN MYELODYSPLASTISCH SYNDROOM (MDS) OP DE KINDERLEEFTIJD

Illustration: MDS, pseudo Pelger-Huet (PB)

De kans om voor de leeftijd van 18 jaar een vorm van kanker te krijgen is ongeveer 1 op 400. Dit betekent dat in Nederland ieder jaar bij ongeveer 600 kinderen een vorm van kanker wordt vastgesteld. De meest voorkomende vorm van kanker op de kinderleeftijd is leukemie, waar verschillende vormen van bestaan. De meest voorkomende vorm is acute lymfatische leukemie, gevolgd door acute myeloïde leukemie (AML). Er zijn ook veel zeldzamere vormen van leukemie of voorstadia daarvan. In dit proefschrift worden twee van deze zeldzame vormen van leukemie beschreven, namelijk Juveniele Myelomonocytaire leukemie (JMML) en Myelodysplastisch Syndroom (MDS). JMML wordt in Nederland gemiddeld 3 keer per jaar vastgesteld en MDS bij gemiddeld 5 kinderen per jaar. Het zijn kwaadaardige afwijkingen van de bloedvormende stamcel, welke leiden tot abnormale bloedcelvorming. Ze kunnen overgaan in een agressieve vorm van leukemie. De enige behandeling met kans op genezing die tot op heden beschikbaar is voor kinderen met JMML en MDS, is stamceltransplantatie. Om een ingang te vinden voor nieuwe behandelingsmogelijkheden is meer kennis nodig over de biologie van JMML en MDS. In dit onderzoek worden moleculaire en genetische afwijkingen van de bloedcellen onderzocht, die aanleiding geven voor het ontstaan van MDS en JMML.

JUVENIELE MYELOMONOCYTAIRE LEUKEMIE

Bij JMML is sprake van een ongecontroleerde celdeling waardoor de gezonde cellen die in het beenmerg aanwezig zijn in de verdrukking komen. Hierdoor ontstaat bloedarmoede, een vergrote gevoeligheid voor infecties en een verhoogde bloedingsneiging. Ook kunnen de lever en milt vergroot zijn door een ophoping van leukemiecellen.

In het eerste deel van dit proefschrift onderzochten we de verschillende aspecten van de moleculaire achtergrond van JMML. In hoofdstuk 1 wordt een overzicht gegeven van moleculaire afwijkingen, die bij een groot aantal van de kinderen met JMML de ongecontroleerde celdeling veroorzaken. Door deze afwijkingen wordt een bepaalde eiwit groep in de cel, de zogenaamde RAS-eiwit groep, geactiveerd. We weten nu dat in ongeveer 85% van de gevallen er een DNA afwijking (mutatie) aanwezig is in de genen die coderen voor een eiwit van deze groep: in 35% van de gevallen een *PTPN11* mutatie, in 25% een *RAS* mutatie en in 11% van de gevallen is er een associatie met Neurofibromatose type I. Een van de andere genen van deze eiwitgroep, het zogenaamde *BRAF* gen (hoofdstuk 2), lijkt bijzonder genoeg niet gemuteerd te zijn bij deze ziekte JMML. Meer recent is aan de hand van klinische observaties van een specifieke vorm van JMML vastgesteld, dat in 17% van de gevallen een afwijking in het *c-CBL* gen wordt gevonden. In ongeveer 15% van de gevallen weten we niet welk gen een rol speelt bij de ontstaanswijze van JMML. Daarnaast beschrijven we in hoofdstuk 1 dat de aanwezigheid van deze afwijkingen niet alleen helpt om het agressieve beloop van de ziekte te begrijpen, maar ook om de diagnose te stellen. In hoofdstuk 3 en 4 hebben we gekeken naar andere genen die mogelijk een rol zouden kunnen spelen bij het ontstaan van JMML.

In hoofdstuk 3 rapporteren we over FLT3 studies in JMML. In bepaalde vormen van AML is dit gen erg actief, waardoor er meer eiwit wordt geproduceerd. Deze overactivatie wordt veroorzaakt door afwijkingen in het DNA van het *FLT3* gen. Dit gen codeert voor een receptor die de RAS-eiwit groep kan activeren. In JMML vonden wij geen bewijs voor overproductie van het FLT3 eiwit of voor de aanwezigheid van mutaties in het *FLT3*

gen. Deze kennis leidt tot de veronderstelling dat FLT3 remmers (reeds beschikbaar als behandeling) niet zinvol zijn bij de behandeling van JMML.

In hoofdstuk 4 beschrijven we de studies over het *WT1* gen bij JMML. We vonden overproductie van het WT1 eiwit wanneer we dit vergeleken met gezonde controlepersonen, maar een lagere productie in vergelijking met patiënten met AML. In 4% van de JMML patiënten toonden we een afwijking in het DNA van het *WT1* gen aan, maar dit was niet geassocieerd met de overproductie van het eiwit. Een vaak voorkomende variant in het DNA van het *WT1* gen is bij kinderen met AML geassocieerd met een grote kans op een slechte overleving. We vonden deze variant regelmatig bij de JMML patiënten, maar niet gerelateerd aan een slechtere uitkomst. We concluderen dan ook dat het *WT1* gen waarschijnlijk geen belangrijke rol heeft bij het ontstaan van JMML, waardoor reeds beschikbare therapie, de zogenaamde immunotherapie, gericht tegen dit gen geen behandeloptie lijkt bij JMML patiënten.

Tot nu toe is beenmergtransplantatie de enige behandeloptie voor genezing van JMML. Gezien het feit dat gezinnen steeds kleiner worden met meestal maar 1 of 2 kinderen, is de kans klein dat er een geschikte donor binnen het gezin wordt gevonden. Wanneer een jonge moeder zwanger is op het moment van de diagnose van JMML bij een van de andere kinderen, kan een transplantatie met navelstrengbloed overwogen worden als er sprake is van een overeenkomende weefselbloedgroep van het pasgeboren kind. Aangezien aan de zogenaamde afstotingsreactie tegen de leukemiecellen (= graft-versus-leukemia) bij een stamceltransplantatie (SCT) bij JMML een belangrijke bijdrage wordt toegekend aan de genezing, is echter de vraag of een SCT met stamcellen van een pasgeboren immuun naïeve baby even effectief is als een SCT met stamcellen van een ouder kind. Daarom hebben wij de effectiviteit van een dergelijke procedure bestudeerd in de Europese registratie van JMML patiënten. Dit liet zien dat navelstrengbloed van een identieke pasgeboren broer of zus een goed alternatief kan zijn voor een transplantatie met beenmerg van een donor uit de beenmergbank.

MYELODYSPLASTISCH SYNDROOM

MDS is een ziektebeeld met verschillende gezichten. De symptomen op het moment van het stellen van de diagnose kunnen erg lijken op die van acute leukemie: bloedarmoede, verhoogde gevoeligheid voor infecties en/of bloedingsneiging. Tot nu toe is er slechts weinig informatie beschikbaar over de ontstaanswijze van MDS op de kinderleeftijd. Het tweede deel van dit proefschrift beschrijft studies, die we hebben uitgevoerd om inzicht te krijgen in de biologische eigenschappen van deze ziekte op de kinderleeftijd.

In hoofdstuk 6 bestudeerden we het optreden van moleculaire afwijkingen in 107 kinderen met MDS. De incidentie van moleculaire en genetische veranderingen (type I en II afwijkingen) bleek veel lager te zijn dan bij volwassenen met MDS. Het was vooral opvallend dat de frequentie van afwijkingen in bepaalde genen die een rol spelen bij het aflezen van de DNA code, opvallend veel lager is dan bij volwassenen met MDS. Dit betekent dat veroudering het optreden van deze moleculaire afwijkingen beïnvloedt.

Met behulp van een nieuwe techniek waarmee kleine afwijkingen in chromosomen kunnen worden aangetoond, zagen we dat meerdere patiënten met MDS met een afwijking op chromosoom 6 een gemeenschappelijke afwijking hadden in een bepaald gen, het *IER3* gen (hoofdstuk 6). Bij volwassenen is vastgesteld dat dit gen een rol speelt

bij het ontstaan van MDS. Wij toonden bij kinderen aan dat de hoeveelheid eiwit dat door dit gen wordt geproduceerd wordt laag was en dat dit met een slechte uitkomst geassocieerd was. We konden echter niet aantonen waardoor deze verlaagde productie werd bepaald.

Een niet eerder beschreven oorzakelijke factor bij het ontstaan van kinderMDS zijn afwijkingen in het mitochondrieel DNA (mtDNA). Mitochondriën spelen een essentiële rol bij de energieproductie van de cel en bezitten hun eigen DNA. Wij vonden bij 68% van de kinderen met MDS een afwijking in het mtDNA. Deze frequentie ligt hoger dan bij volwassenen. Onze hypothese is dat bij kinderen mtDNA mutaties in de bloedvormende stamcel leiden tot een verhoogde gevoeligheid voor het ontwikkelen van additionele afwijkingen, de zogenaamde tweede hit. Mogelijk spelen mtDNA mutaties ook een rol bij het ontstaan van andere vormen van kinderkanker.

In hoofdstuk 9 wordt de rol van auto-immuniteit bij het ontstaan van een specifieke vorm van MDS beschreven, de zogenaamde refractaire cytopenie. Dit ziektebeeld is moeilijk te onderscheiden van een andere beenmergaandoening, te weten aplastische anemie (AA). Bij een deel van de kinderen met MDS lijkt net zoals bij AA, auto-immuniteit tegen vroege voorloper stamcellen op te treden. We onderzochten de aanwezigheid van T-cel receptor Vβ oligoklonaliteit bij kinderen met refractaire cytopenie en aplastische anemie. De frequentie van deze zogenaamde auto-immuniteit is zowel bij refractaire cytopenie als aplastische anemie 60%, hetgeen duidelijk maakt dat deze test niet gebruikt kan worden om beide ziektes van elkaar te onderscheiden. Het onderstreept echter de hypothese dat bij een subgroep van patiënten met MDS de verstoringen in het immuunsysteem verantwoordelijk zijn voor het ontstaan van de MDS en dat deze patiënten mogelijk baat kunnen hebben bij een behandeling met immuunsuppresieve therapie.

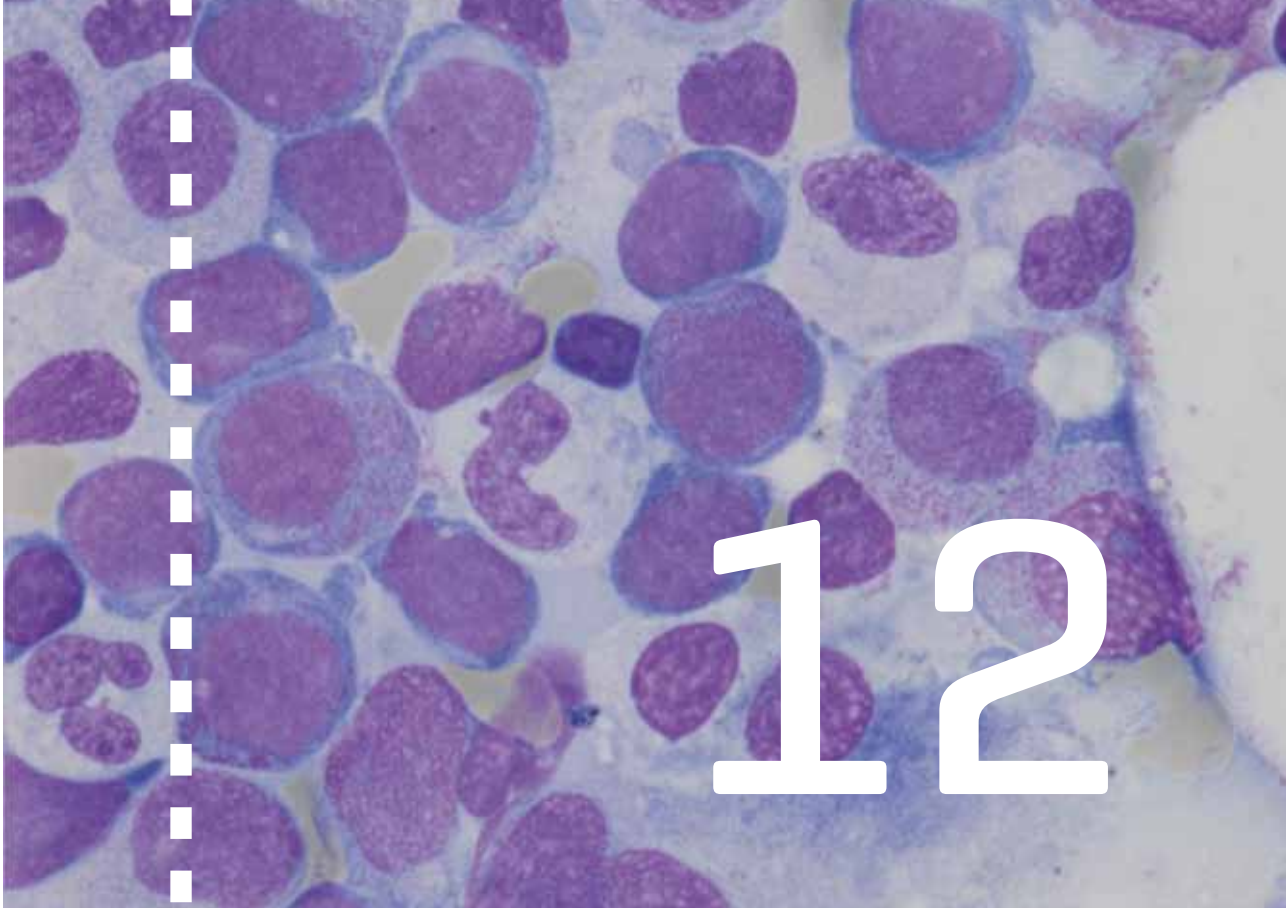

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Illustration: MDS, dysmyelopoiesis and excess of blasts [RAEB] [BM]

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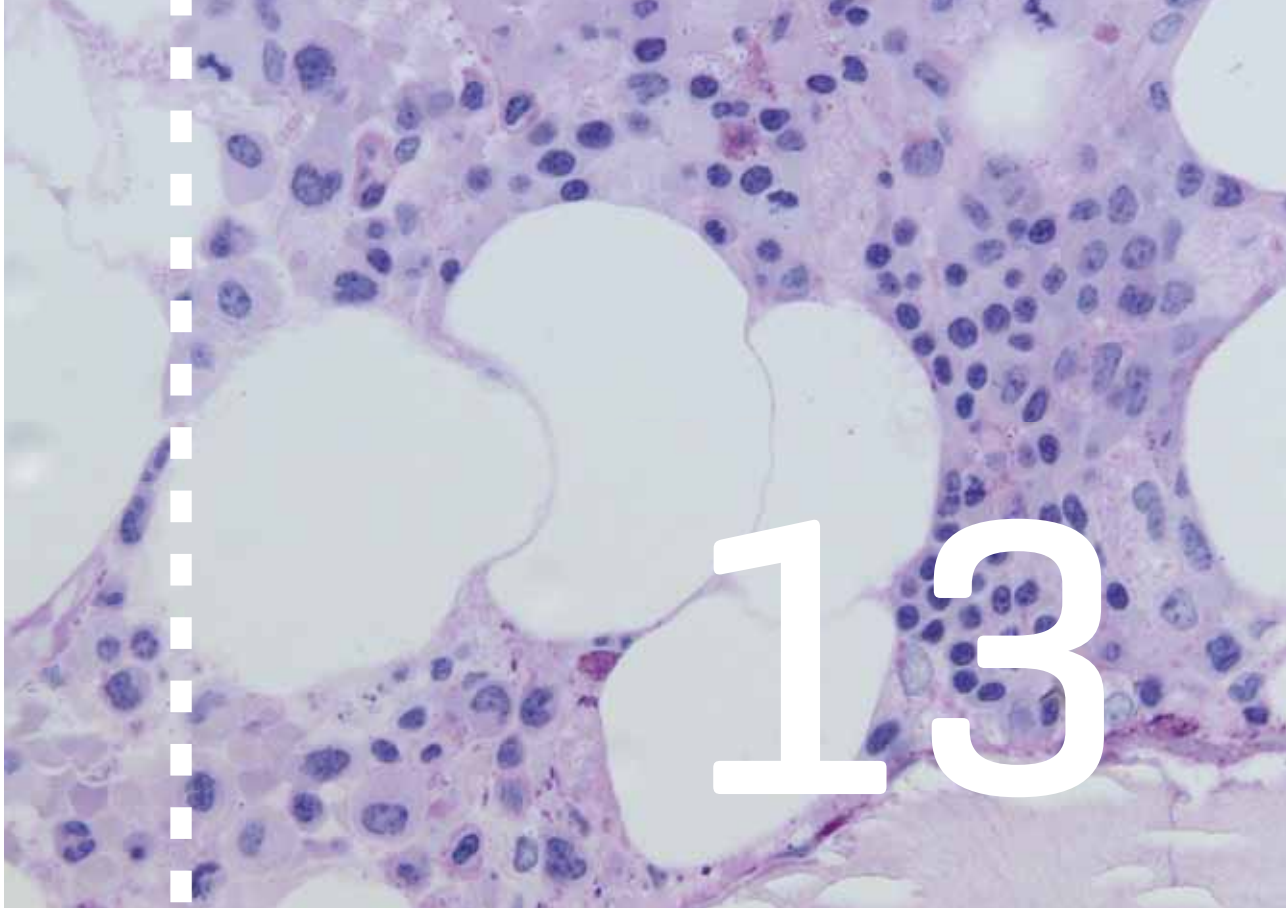

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ABOUT THE AUTHOR

Illustration: MDS, dysplasia in erythropoiesis and hypocellular bone marrow

CURRICULUM VITAE

Andrica de Vries werd op 14 maart 1970 geboren te Leeuwarden. Na het behalen van haar VWO diploma aan het Bogerman College te Sneek ging zij Geneeskunde studeren aan de Rijksuniversiteit Groningen. Tijdens haar doctoraal fase volgde zij bijvakken bij de faculteit Godgeleerdheid, gericht op Medische Ethiek. Dit resulteerde in een wetenschappelijke stage over actieve levensbeëindiging bij niet terminaal zieke mensen (supervisor dr. T. Tijmstra, Medisch Socioloog, Rijksuniversiteit Groningen).

Zij deed haar coschappen in de Deventer Ziekenhuizen te Deventer. Haar keuze-coassistentschap bestond uit een stage van vijf maanden op de afdeling kinderhemato-oncologie van het Sophia Kinderziekenhuis in Rotterdam (toenmalig hoofd: dr. K. Hählen). Zij behaalde haar artsexamen in 1996.

Vervolgens was zij werkzaam als arts-assistent kindergeneeskunde op de afdeling hemato-oncologie van het Sophia Kinderziekenhuis, het Groene Hart Ziekenhuis te Gouda en op de medium care van het Sophia Kinderziekenhuis. In 1999 startte zij met de opleiding tot kinderarts in het Sophia kinderziekenhuis, welke zij in 2004 succesvol afrondde (opleider: prof.dr. H.A. Büller).

Daarna was zij werkzaam als chef de clinique algemene kindergeneeskunde op de medium care kindergeneeskunde, waarna zij in 2004 startte met de opleiding tot kinderhemato-oncoloog (hoofd: prof.dr. R. Pieters). Tijdens dit fellowship werd de grondslag voor dit proefschrift gelegd. Tevens was zij enkele maanden werkzaam op de afdeling kinderoncologie en stamceltransplantatie van de Universiteit van Freiburg in Freiburg, Duitsland (hoofd: prof.dr. C.M. Niemeyer), om kennis te vergaren over beenmergtransplantatie, MDS en JMML.

Sinds 2007 is zij werkzaam als staflid binnen de kinderhemato-oncologie van het ErasmusMC Sophia Kinderziekenhuis en verrichte zij het onderzoek zoals beschreven in dit proefschrift. Momenteel is zij een actief lid van de EWOG-MDS (European Working Group of MDS and JMML in Childhood), ziektecommissies MDS en aplastische anemie van het SKION (Stichting Kinderoncologie Nederland) en de protocolcommissie Post-Transplantation Lymphoproliferative Disease (PTLD). Verder is zij aangesteld als kinderarts bij de LATER (LAnge TERmijn effecten) polikliniek en is zij chef de clinique op de afdeling Kinderoncologie.

Zij is gehuwd met Kees Bosch en samen hebben zij drie kinderen: Elke (2004), Hanne* (2006) en Ward (2008).

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1. PHD TRAINING

GENERAL COURSES

2010-2011	VVAA: kadertraining
2009	Biomedical English Writing and Communication
2009	Course of Molecular Diagnostics IV
2009	Course Presenting and Information Transfer
2009	Course Selfreflection and Management
2008	Effective Presentation of Scietific Research
2007	Classical Methods for Data Analysis (CCO2) (NIHES)

SPECIFIC COURSES

2009	ASPO Masterclass, Amsterdam School of Pediatric Oncology
2008	ASPO 4th Postgraduate Corse in Pediatric Oncology, Amsterdam School of Pediatric Oncology
2006	ASPO 3th Postgraduate Corse in Pediatric Oncology, Amsterdam School of Pediatric Oncology
2006	Basic and Translational Oncology

SEMINARS AND WORKSHOPS

2009	Annual PhD day, ErasmusMC
2009	Annual Pediatric Research day, ErasmusMC
2008	Day Young Pediatric Researchers, NVK, Veldhoven
2007-2010	Annual Pediatric Oncology Symposium, ErasmusMC

ORAL PRESENTATIONS

JMML versus CMML in adults

2012	4th Symposium on Hematomorphology, Rotterdam

Mitochondrial DNA mutations are involved in the development of childhood MDS

2012	Bi-annual Leukemia meeting, Santiago, Chili

Molecular aberrations in 107 children MDS

2012	Bi-annual Leukemia meeting, Santiago, Chili

Juvenile Myelomonocytic Leukemia in twins

2011	EWOG-MDS-AA, business meeting, Ghent, Belgium
2011	JMML Foundation meeting, San Diego, United States

Myelodysplastic syndrome in children: is it the same as in adults?
2011 3rd Symposium on Hematomorphology

Myelodysplastic Syndrome and Juvenile Myelomonocytic Leukemia
2010-2012 Minor Pediatric Oncology, ErasmusMC, Rotterdam

Myelodysplastic Syndrome and Juvenile Myelomonocytic Leukemia
2010 Grand Round, ErasmusMC, Rotterdam

Role of WT1 in the pathogenesis of JMML
2010 JMML foundation meeting, New Orleans, United States

Chromosome 6 aberrations in childhood MDS
2009 2nd Workgroup and 3rd Management Committee meeting of the EuGESMA, European Genetic and Epigenetic Study on MDS and AML, Hannover, Germany

HLA-identical umbilical cord blood transplantation from a sibling donor in juvenile myelomonocytic leukemia
2009 10th International symposium on myelodysplastic syndromes, Patras, Greece

Pediatric myelodysplastic syndrome in a family with mitochondrial DNA mutations
2009 5th International Congress of the European Working Group on Myelodysplastic Syndromes and Bone Marrow Failures, Rotterdam, The Netherlands

BRAF mutations in juvenile myelomonocytic leukemia
2008 JMML foundation meeting, San Francisco, United States

Role of mutation independent constitutive activation of *FLT3* in juvenile myelomonocytic leukemia
2006 4th International Symposium on MDS in Childhood, Freiburg, Germany
2008 JMML foundation meeting, San Francisco, United States

POSTER PRESENTATIONS

Role of mitochondrial DNA mutations in childhood MDS
2011 11th International Symposium on Myelodysplastic Syndromes, Edinburgh, UK
2011 ASH, San Diego, United States

IER3 in childhood MDS
2011 11th International Symposium on Myelodysplastic Syndromes, Edinburgh, UK
2011 ASH, San Diego, United States

HLA-identical umbilical cord blood transplantation from a sibling donor in juvenile myelomonocytic leukemia
2009 5th International Congress of the European Working Group on 2009 Myelodysplastic Syndromes and Bone Marrow Failures, Rotterdam, The Netherlands

Pediatric myelodysplastic syndrome in a family with mitochondrial DNA mutations

2009 10th International symposium on myelodysplastic syndromes, Patras, Greece

BRAF mutations in Juvenile Myelomonocytic Leukemia

2007 EHA, Vienna, Austria

2007 9th International symposium on myelodysplastic syndromes, Florence, Italy

T-cell repertoire Vβ CDR3 oligoclonality frequently occurs in childhood refractory cytopenia

2007 EHA, Vienna, Austria

2007 9th International symposium on myelodysplastic syndromes, Florence, Italy

2007 ASH, Atlanta, United States

AWARDS

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Pediatric myelodysplastic syndrome in a family with mitochondrial DNA mutations

2009 10th International symposium on myelodysplastic syndromes, Patras, Greece

2. TEACHING

2010-2012 Supervising Minor students

PUBLICATIONS

A.C.H. de Vries, C.M. Zwaan, I.H.I.M. Hollink, A. Willasch, M. Alders, V. de Haas, M.N. Dworzak, H. Hasle, F. Locatelli, M. Zecca, B. De Moerloose, J. Stary, C. Flotho, C.M. Niemeyer, P. Bader, R. Pieters, M.M. van den Heuvel-Eibrink, Relevance of *WT1* expression, mutations and single nucleotide polymorphisms in Juvenile Myelomonocytic Leukemia, *submitted*

A.C.H. de Vries, C.M. Zwaan, J.H. Jansen, M.N. Dworzak, H. Hasle, F. Locatelli, B. De Moerloose, T.J.C.M. Arentsen-Peters, V. de Haas, S. Polychronopoulou, J. Stary, M. Schmugge-Liner, M. Zecca, O. Smith, A. Catala, B. Schlegelberger, H.B. Beverloo, R. Pieters, C.M. Niemeyer, M.M. van den Heuvel-Eibrink, an EWOG–MDS study, Molecular aberrations in 107 children with myelodysplastic Syndrome (MDS), *submitted*

A.C.H. de Vries, C.M. Zwaan, A. Danen-van Oorschot, V. de Haas, H. Hasle, F. Locatelli, B. De Moerloose, S. Polychronopoulou, J. Stary, M. Schmugge-Liner, M.N. Dworzak, M. Zecca, H.B. Beverloo, C.M. Niemeyer, R. Pieters, M.M. van den Heuvel-Eibrink, *IER3* expression in childhood MDS, *submitted*

A.C.H. de Vries, C.M. Zwaan, H.B. Beverloo, A. Wagner, A.C. Lankester, P.A.W. te Boekhorst, I.F.M. de Coo, G.C. Schoonderwoerd, D.M.E.I. Hellebrekers, A.T. Hendrickx, H.J.M. Smeets, S. Polychronopoulou, R. Pieters, M.M. van den Heuvel-Eibrink, Mitochondrial DNA mutations are involved in the development of childhood Myelodysplastic Syndrome, *submitted*

I.M.M. van der Geest, W. van Dorp, W.C.J. Hop, S.J.C.M.M. Neggers, **A.C.H. de Vries**, R. Pieters, F.K. Aarsen, M.M. van den Heuvel-Eibrink, Emotional distress in 652 Dutch very long-term survivors of childhood cancer, using the Hospital Anxiety and Depression Scale (HADS), *submitted*

W. van Dorp, I.M.M. van der Geest, J.S.E. Laven, W.C.J. Hop, S.J.C.M.M. Neggers, **A.C.H. de Vries**, R. Pieters, M.M. van den Heuvel-Eibrink, Gonadal function recovery in very long-term male survivors of childhood cancer, *submitted*

M. de Mol, J.C. de Jongste, M. van Westreenen, P.J. Merkus, **A.C.H. de Vries**, W.C. Hop, A. Warris, H.M. Janssens, Diagnosis of invasive pulmonary aspergillosis in children with bronchoalveolar lavage galactomannan, Pediatr Pulmonol. 2012, epub ahead of print

A.C.H. de Vries, C.M. Zwaan, M.M. van den Heuvel-Eibrink, Molecular basis of juvenile myelomonocytic leukemia, Haematologica, 2010;95(2):179-82

A.C.H. de Vries, B. De Moerloose, E.R. van Wering, M.M. van den Heuvel-Eibrink, Myelodysplatisch syndroom en juveniele myelomonocytaire leukemie, Nederlands Tijdschrift voor Hematologie, 2009; 6: 63-70

A.C.H. de Vries, R.G.M. Bredius, A.C. Lankester, M. Bierings, M. Trebo, P. Sedlacek, C.M. Niemeyer, M. Zecca, F. Locatelli, M.M. van den Heuvel-Eibrink, HLA identical umbilical cord blood transplantation from a sibling donor in juvenile myelomonocytic leukaemia, Haematologica, 2009 Feb;94(2):302-4.

A.C.H. de Vries, A.W. Langerak, B. Verhaaf, C.M. Niemeyer, J. Stary, K. Schmiegelow, E.R. van Wering, C.M. Zwaan, A. Beishuizen, R. Pieters, M.M. van den Heuvel-Eibrink, T-cell receptor Vbeta CDR3 oligoclonality frequently occurs in childhood refractory cytopenia and severe aplastic anemia, Leukemia, 2008 Jun;22(6):1170-4.

A.C.H. de Vries, R.W. Stam, C.P. Kratz, M. Zenker, C.M. Niemeyer, M.M. van den Heuvel-Eibrink, Mutation analysis of the *BRAF* oncogene in juvenile myelomonocytic leukemia, Haematologica, 2007 Nov;92(110:1574-5.

I.M. Onvlee-Dekker, **A.C.H. de Vries,** D.J. ten Harkel, Carbon monoxide poisoning mimicking long QT induced syncope, Arch Dis Child. 2007 Mar;92(3):244-5.

A.C.H. de Vries, R.W. Stam, P. Schneider, C.M. Niemeyer, E.R. van Wering, O.A. Haas, C.P. Kratz, M.L. den Boer, R. Pieters, M.M. van den Heuvel-Eibrink, Role of mutation independent constitutive activation of FLT3 in juvenile myelomonocytic leukaemia, Haematologica, 2007 Nov;92(11):1557-60.

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Andrica