Molecular Insights in *MLL* Rearranged Acute Leukemia

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Molecular Insights in *MLL* Rearranged Acute Leukemia

Moleculaire inzichten in *MLL* herschikte acute leukemie

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"I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Sir Isaac Newton (1642 - 1727)

Voor Saskia
Owen en Caitlin

CONTENTS

Chapter 1

General Introduction

1.1. LEUKEMIA

– In science the credit goes to the man who convinces the world, not to the man to whom the idea first occurs. –
William Osler (1849–1919) Canadian physician.

In 1845 both the British pathologist John Hughes Bennett and the German pathologist Rudolf Virchow described leukemia as a lethal disease characterized by enlargement of the spleen, and an excess of cells in the blood.[1-3] Although both claimed to have been the first to describe leukemia, and current textbooks usually ascribing the discovery of this disease to either one of them, it was in fact the French physician Alfred Donné who published the first description of leukemia in his book *Complimentary course on microscopy for medical studies* in 1844.[4,5] Although merely armed with a microscope, Donné actually believed that the excess of cells he observed in the blood of patients with this mysterious disease was a result of an arrest in maturation of intermediate blood cells.[4,5] Nowadays we know that Donné could not have been more right, as leukemia (Greek for "white blood") is a type of cancer that is characterized by uncontrolled accumulation of immature white blood cells in the bone marrow.

Normally, the bone marrow is responsible for the formation of new blood cells (hematopoiesis), which is a tightly balanced and highly organized process of proliferation, differentiation, maturation, and cell survival. In case of leukemia, malignant cells eventually replace the entire population of healthy blood cells, resulting in anemia (low number of red blood cells), internal bleeding (low number of platelets), and increased susceptibility to infections (low number of normal white blood cells). Ultimately, leukemic cells enter the peripheral blood circulation and infiltrate other organs like the spleen, liver, and kidneys. When left untreated, leukemia is a lethal disease.

1.1.2. Types of leukemia

Like *cancer* is not simply ***a*** disease, but rather a generic term for over a hundred of different diseases, *leukemia* is a collective noun for over a dozen of different leukemias. First and foremost, different types of leukemia are defined by the type of blood cell that was subject to malignant transformation. Driven by cytokines or hematopoietic growth factors, all types of blood cells develop from a communal pool of self-renewing pluripotent hematopoietic stem cells (HSCs). These pluripotent HSCs are able to differentiate towards either lymphoid or non-lymphoid committed stem cells. Lymphoid stem cells have the ability to differentiate and mature into either B- or T-lymphocytes, whereas non-lymphoid stem cells eventually differentiate towards myeloid cells like granulocytes (i.e. neutrophils, eosinophils, and basophils), monocytes, and macrophages, or towards megakaryocytes, platelets and erythrocytes. Depending on

the lineage of the cell in which the leukemia originates, leukemia can be divided into lymphoid (or lymphocytic) and non-lymphoid (or myeloid) leukemia. Accordingly, lymphoid leukemias can be subdivided into B- and T-cell leukemias. Likewise, based upon the type of non-lymphoid cell that is affected, several subgroups of non-lymphoid (or myeloid) leukemias can be identified.

Both lymphoid and myeloid leukemias can further be classified into *acute* and *chronic* leukemias. Acute leukemias are rapidly progressing leukemias involving highly immature hematopoietic progenitors that are differentiated only limitedly. In contrast, chronic leukemias involve well-differentiated (but immunologically incompetent) cells, and usually develop more slowly.

1.1.3. Childhood acute lymphoblastic leukemia (ALL)

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children, representing about one quarter of all childhood cancers. Annually, 3-4 per 100.000 children are diagnosed with this type of leukemia. In approximately 75% of the childhood ALL cases, the leuke mic cells are characterized by recurrent chromosomal abnormalities, including balanced chromosomal translocations and numerical anomalies.[6] Based on these recurrent abnormalities, ALL can even be further subdivided into a variety of genetic subtypes, all with distinct etiologies, biological and clinical features, treatment outcomes, and genetic constitutions.[6,7] Fortunately, the prognosis for childhood ALL in general nowadays is favorable, with approximately 80% of the children treated with combination chemotherapy surviving their disease.[6]

1.2. INFANT ACUTE LYMPHOBLASTIC LEUKEMIA

Unfortunately, for some subgroups of patients the prognosis still remains poor. In approximately 4% of the childhood cases, ALL is diagnosed in an infant (i.e. a child less than 1 year of age). Infants with ALL form the most striking example of a subgroup of ALL patients who did not benefit from the greatly improved treatment regimens. Although complete remission (CR) is achieved in the vast majority (~95%) of these very young children[8,9], a good outcome usually is hampered by an exceedingly high relapse rate, typically occurring within the first year upon diagnosis. Consequently, the overall survival for infant ALL patients to date remains a dismal 40-50%.[10,11]

1.2.1. *MLL* gene rearrangements and infant ALL

ALL in infants is characterized by an exceptionally high incidence of leukemia specific chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*, *ALL-1* or *HRX*) gene, which occur in approximately 80% of the cases.[12,13] Such chromosomal

translocations arise during illegitimate recombination events that result in the interchange of fragments between non-homologous chromosomes. The *MLL* gene comprises a so-called breakpoint cluster that behaves like a hot-spot for chromosomal translocations which reciprocally fuse the N-terminal portion of the *MLL* gene to the C-terminal region of one of its translocation partner genes (Figure 1), of which over 50 have been described.[14] Remaining in-frame, these gene fusions encode chimeric transcripts which give rise to oncogenic fusion proteins with pronounced transforming potential (extensively reviewed in ref.15,16). The genesis of these *MLL* fusion proteins are believed to be initiating events in the development of infant ALL, and take place *in utero* (see chapter 2). By far the most common *MLL* rearrangements found in infant ALL patients are translocations t(4;11) (~50%), t(11;19) (~20%) and t(9;11) (~10%), fusing the *MLL* gene to the transcription factors *AF4*[17], *ENL*[18] and *AF9*[19] respectively.

1.2.2. The immunophenotype of infant ALL

Leukemic cells from Infant ALL patients carrying *MLL* rearrangements typically display an immature CD19-positive ($CD19^+$), CD10 negative ($CD10^-$) precursor B-lineage (pro-B) immunophenotype, and are often characterized by co-expression of myeloid-associated antigens like CD15 and CDw65. Mature B-cell or T-cell phenotypes are observed only sporadically[20,21]. In contrast, infant ALL cells carrying germ line *MLL* genes far more often resemble common ($CD19^+$,$CD10^+$) or pre-B ($CD19^+$,$CyIg\mu^+$) immunophenotypes.

1.2.3. Prognostic factors in infant ALL

Among many prognostic factors identified for infant ALL, including the presence of translocations involving *MLL*, age less than 6 months, high white blood cell counts, lack of CD10 expression, myeloid antigen co-expression, central nervous system involvement, and a poor *in vivo* response to prednisone, the presence of *MLL* rearrangements seems to be the most important independent predictor for an adverse outcome (see chapter 2). Several studies have demonstrated that particularly those infant ALL cases carrying *MLL* rearrangements experience a poor prognosis, whereas the prognosis for infant ALL patients bearing germ line *MLL* genes is much more favorable, with long-term survival rates easily exceeding 60%.[22-26]

1.2.4. Cellular drug resistance in infant ALL

Considerably contributing to the dismal prognosis of the majority of infant ALL patients seems to be cellular drug resistance. Leukemic cells from infant ALL patients are significantly more resistant *in vitro* to several important chemotherapeutic drugs compared to cells from older children with ALL, especially to glucocorticoids (prednisone and dexamethasone) and L-asparaginase.[27,28] Since the *in vitro* and *in vivo* response to

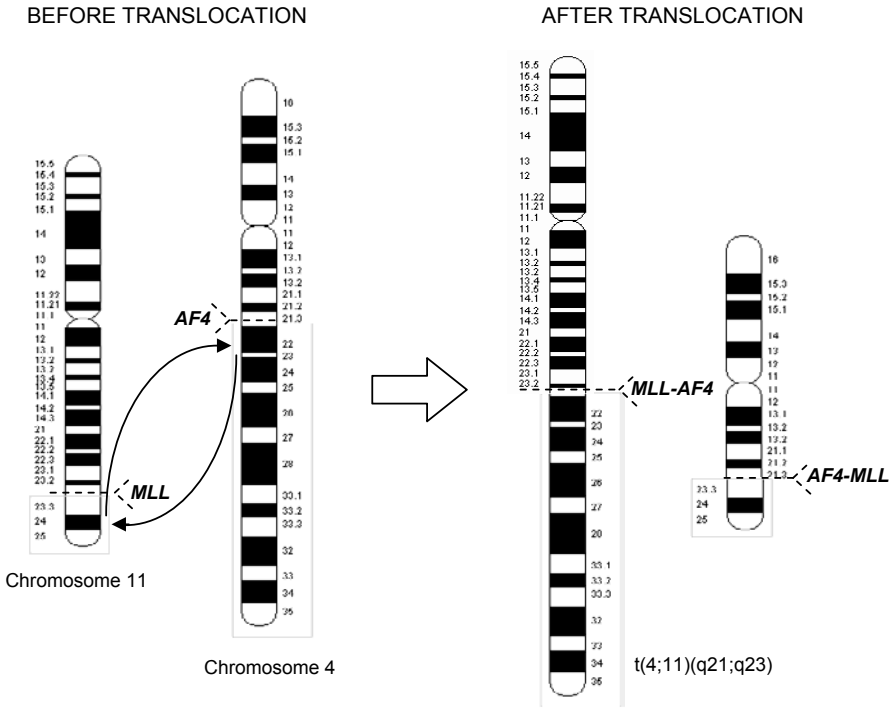

Figure 1. *MLL* rearrangement.
Schematic representation of chromosomal translocation t(4;11)(q21;q23), fusing the N-terminal region of the *MLL* gene on chromosome 11 to the C-terminal region of the *AF4* gene on chromosome 4, and vice versa.

prednisone is highly predictive for clinical outcome in childhood ALL in general[25,29,30], the poor prognosis for infant ALL may to a large extent be associated with the observed resistance to glucocorticoids, and presumably with resistance to other drugs as well. Thus, with current treatment protocols for childhood ALL failing in over 50% of the infant ALL cases, more effective (innovative) therapeutic strategies are urgently needed in order to improve prognosis for this aggressive type of leukemia.

Although relatively resistant to several important chemotherapeutic drugs, infant ALL cells appeared to respond remarkably well to the cytidine analogue Ara-C (cytosine arabinoside). Infant ALL cells are significantly more sensitive to Ara-C as compared to cells from older children with ALL.[27,28] This observation suggested that infant ALL might resemble a subclass of childhood ALL that may well benefit from intensified treatment with Ara-C. Therefore, in 1999 a novel collaborative treatment protocol (INTERFANT-99) was developed with intensified use of Ara-C throughout the protocol, in order to provide a more specific treatment for infant ALL patients.

1.3. OUTLINE OF THIS THESIS

Chapter 2 of this thesis comprises a review describing important aspects of infant ALL, including *MLL* rearrangements, the cell of origin, the prenatal origin of this type of leukemia, the etiology and risk factors, prognostic factors, cellular drug resistance, and putative therapeutic approaches to improve prognosis (mostly based on studies described elsewhere in this thesis).

Chapter 3 describes a study investigating the mechanism underlying the remarkable sensitivity of infant ALL cells to Ara-C. This study demonstrates that elevated expression of the gene encoding the human equilibrative nucleoside transporter 1 (*ENT1*), on which Ara-C is mainly dependent to permeate the cell membrane, provides a plausible explanation for this phenomenon. Since Ara-C is a drug that is typically used in the treatment of acute myeloid leukemia (AML), and *MLL* rearranged infant ALL cells often display myeloid characteristics, a reasonable hypothesis would be that Ara-C sensitivity (as a result of increased *ENT1* expression) is associated with the presence of *MLL* rearrangements. In **chapter 4** we address this hypothesis, and show that there is no direct association of the presence of *MLL* rearrangements and sensitivity to Ara-C. In **chapter 5** we investigated whether the mechanism underlying Ara-C sensitivity in infant ALL (i.e. increased *ENT1* expression), also applies to childhood AML. This study revealed that elevated expression of *ENT1* in childhood AML samples not only predicts sensitivity to Ara-C, but also appeared to be associated with sensitivity towards other nucleoside analogue drugs such as cladribine, decitabine, and gemcitabine.

Since infant ALL cells *in vitro* are resistant to multiple chemotherapeutic drugs[27,28], infant ALL patients may legitimately be classified as multidrug resistant. Therefore, a plausible explanation for the chemo-resistant character of infant ALL cells could be the involvement of multidrug resistance proteins that function as specialized membrane pumps capable of trafficking chemotherapeutic drugs out of the cell. In **chapter 6** we investigated whether drug resistance in infant ALL is a consequence of increased drug efflux mediated by multidrug resistance pumps.

Given the poor response of infant ALL patients to current chemotherapeutic regimes, it is of utmost importance to explore innovative therapeutic strategies. For this, we set out to search for molecular targets suitable to direct therapy against. In collaboration with Dr. Scott Armstrong (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, USA) we observed that the gene encoding Fms-like tyrosine kinase receptor 3 (*FLT3*) is highly expressed in *MLL* rearranged ALL samples[31]. As a consequence of high-level expression we demonstrated that FLT3 is constitutively activated in an *MLL* rearranged ALL cell line, promoting leukemic cell proliferation and survival.[32] In **chapter 7** we studied whether high-level expression of *FLT3* is associated with constitutive FLT3 signaling in primary *MLL* rearranged infant ALL cells. Moreover, we investigated

whether inhibition of FLT3 using a small molecule FLT3 inhibitor may represent a novel therapeutic approach against this type of leukemia.

Constitutively activated FLT3 also frequently occurs in primary AML cells, predominantly caused by specific mutations within the *FLT3* gene. In **chapter 7** and **chapter 8** we studied whether these activating mutations also occur in *MLL* rearranged infant ALL, and if so, how frequently these genetic abnormalities occur. For this we screened the entire *FLT3* gene for the presence of known and novel mutations, and demonstrate that the main etiology for constitutive FLT3 signaling in *MLL* rearranged infant ALL cells is over-expression of wild-type *FLT3*.

In addition to exploring the use of over-expressed genes as therapeutic targets for infant ALL, we also studied genes that appeared to be under-expressed in infant ALL as compared to other ALL subtypes. **Chapter 9** describes a study demonstrating that *MLL* rearranged infant ALL is characterized by the silencing of the tumor suppressor gene *FHIT*, and how this phenomenon provides a rationale for the use of demethylating agents as therapeutic intervention for these patients.

Finally, **chapter 10** summarizes the work presented in this thesis, accommodated with general conclusions and future perspectives. **Chapter 11** comprises a concise layman's summary of this thesis in Dutch.

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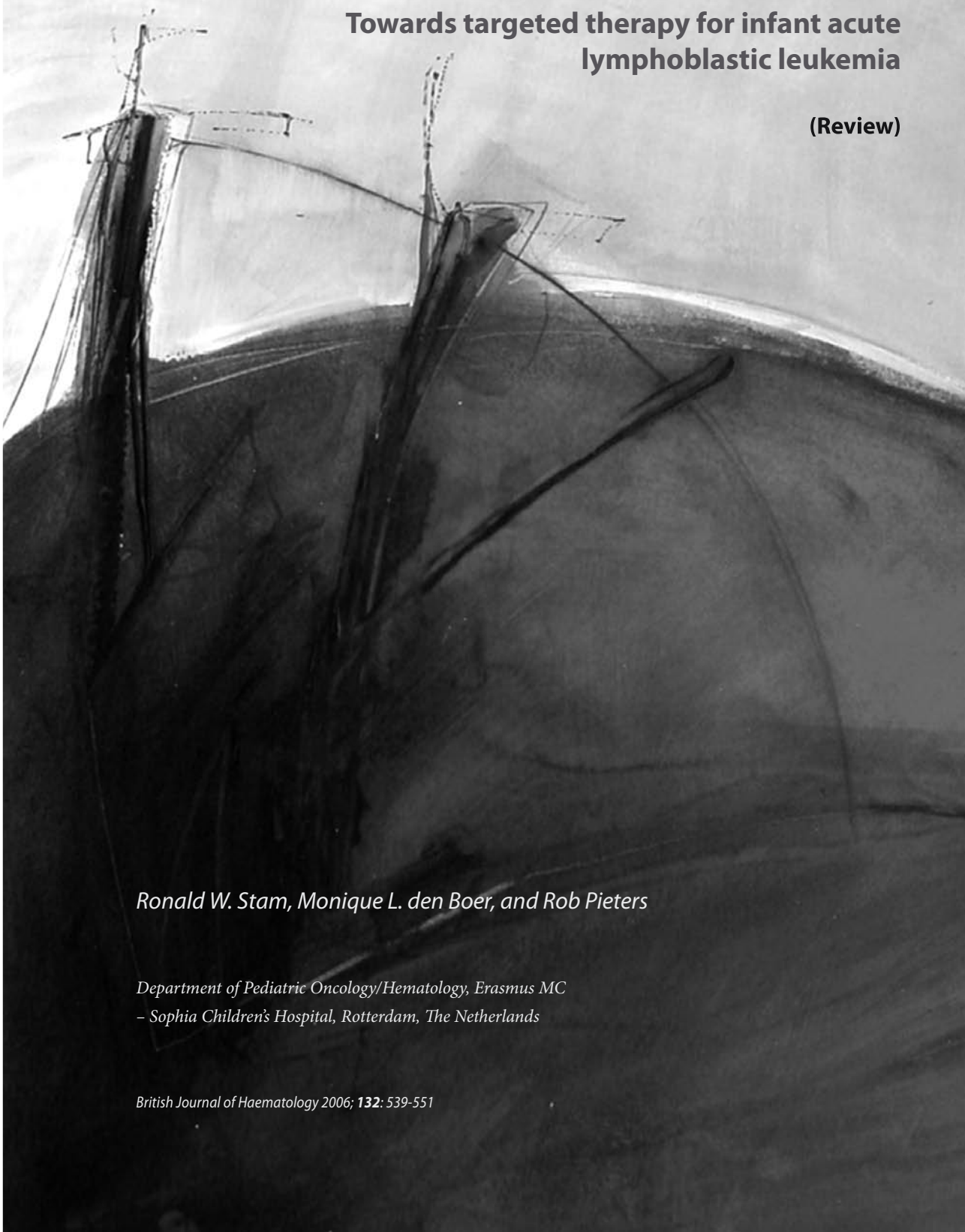
Chapter 2

Towards targeted therapy for infant acute lymphoblastic leukemia

(Review)



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ABSTRACT

Despite the greatly improved treatment regimes for childhood acute lymphoblastic leukemia (ALL) in general, resulting in long-term survival in approximately 80% of cases, current therapies still fail in >50% of ALL cases diagnosed within the first year of life (i.e. in infants). Therefore, more adequate treatment strategies are urgently needed to also improve prognosis for these very young patients with ALL. Here we review the current acquaintance with the biology of infant ALL and describe how this knowledge may lead to innovative therapeutic approaches.

INTRODUCTION

Current treatment strategies for childhood acute lymphoblastic leukemia (ALL) nowadays result in long-term survival in approximately 80% of the cases.[1] Unfortunately, for some subgroups of patients the prognosis still remains poor. Infants (i.e. children less than 1 year of age) form the most striking example of ALL patients who have not benefited from the greatly improved treatment regimens that have developed over recent decades. Although morphological complete remission (CR) is achieved in the vast majority (~95%) of these very young children[2,3], a favorable outcome usually is hampered by an exceedingly high relapse rate, typically within the first year after CR. Consequently, the overall survival for infant ALL patients to date remains at best a dismal 40-50%[4] (Table 1). Therefore, novel therapeutic strategies are urgently needed for this aggressive type of leukemia. Here we review the current knowledge on infant ALL and discuss how this information may translate into therapeutic possibilities that may improve prognosis.

Table 1. Treatment results in infant ALL.

Study group	4yr-EFS	Sample size	Reference
DFCI 85-01	54%	n=23	Silverman, *et al* (1997)[5]
BFM	43%	n=105	Dordelmann, *et al* (1999)[6]
EORTC-CLCG	43%	n=25	Ferster, *et al* (1994)[7]
CCG-1883	39%	n=135	Reaman, *et al* (1999)[3]
CCG-107	33%	n=99	Reaman, *et al* (1999)[3]
UKALL-92	33%	n=86	Chessells, *et al* (2002)[8]
POG 8493	28%	n=82	Frankel, *et al* (1997)[2]
POG (Alternating drugs)	17%	n=33	Lauer, *et al* (1998)[9]

MLL GENE REARRANGEMENTS

Infant ALL is characterized by an exceptionally high incidence of leukemia-specific rearrangements involving the *Mixed Lineage Leukemia* (*MLL*, *ALL-1* or *HRX*) gene on chromosome band 11q23, which occur in about 80% of the cases.[10,11] These chromosomal abnormalities, believed to be initiating events in leukemogenesis, usually involve reciprocal translocations fusing the N-terminal portion of the *MLL* gene to the C-terminal region of one of its translocation partner genes, of which over 50 have been described.[12,13] Remaining in-frame, such gene fusions encode chimeric transcripts which give rise to oncogenic fusion proteins with pronounced transforming potential (extensively reviewed in refs. 14 and 15). By far the most common *MLL* translocations found in infant ALL patients are t(4;11), t(11;19) and t(9;11), fusing *MLL* to the transcription factors *AF4*[16], *ENL*[17] and *AF9*[18] respectively. Among several prognostic factors

identified in infant ALL (described below), the presence of *MLL* rearrangements seems to be the most important independent predictor of an adverse outcome. In particular, infant ALL cases carrying *MLL* rearrangements experience a poor prognosis, whereas the prognosis for infant ALL patients bearing germ line *MLL* genes is much more favorable, with long-term survival rates easily exceeding 60%.[19-22] This, together with recent micro-array studies demonstrating that *MLL* rearranged leukemias display characteristic gene expression profiles that distinguish them from other childhood ALL subtypes[23,24], suggests that *MLL* rearranged ALL represents a unique biological entity that is frequently diagnosed at very young age (i.e. within the first year of life). In this review we will specifically focus on *MLL* rearranged ALL (from here on abbreviated as MLL) in infants.

THE CELL OF ORIGIN

Infant MLL cells typically display an immature CD19-positive ($CD19^+$) and CD10 negative ($CD10^-$) precursor B-lineage (i.e. pro-B) immunophenotype, and are often characterized by co-expression of myeloid-associated antigens like CD15 and CDw65. Mature B-cell or T-cell phenotypes are observed only sporadically.[25,26] In contrast, infant ALL cells carrying germ line *MLL* genes more often resemble common ($CD19^+$,$CD10^+$) or pre-B ($CD19^+$,$CyIg\mu^+$) immunophenotypes. Recently Fais *et al* demonstrated that the expression of CD1d, a monomorphic molecule involved in glycolipid presentation, is associated with infant age (<1 year), the pro-B immunophenotype and the presence of translocation t(4:11).[27]

Co-expression of myeloid markers on highly immature B-cell progenitors in addition to observed lineage switches from ALL to acute myeloid leukemia (AML)[28] and vice versa[29,30] in *MLL* rearranged leukemias, strongly points to a type of B-cell/myeloid progenitor[31,32] as the cell from which infant MLL originates. The characteristic gene expression profile associated with *MLL* rearranged ALL seems to support this (reviewed by Armstrong, *et al*[33]). However, increasing evidence is emerging that the self-renewal properties of certain types of high-risk acute leukemias are sustained by the presence of a minor sub-population of leukemic stem cells from which the leukemia originates. Hotfilder *et al* recently reported the presence of leukemia-specific translocations in primitive lymphoid-restricted $CD34^+CD19^-$ cells purified from primary t(4;11) positive infant ALL samples which predominantly consist of leukemic B-lineage $CD34^+CD19^+$ cells. This indicated that t(4;11) positive ALL actually originates in a primitive lymphoid-restricted progenitor/stem cell[34], which give rise to leukemic cells that, to some extent, retain the ability to differentiate towards the pro-B ($CD34^+CD19^+CD10^-$) compartment. In support of this latter is a study demonstrating that $CD34^+CD19^-$ cells

purified from primary human B-lineage ALL samples injected into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice, induced leukemia *in vivo* with both immunophenotypes and karyotypes similar to that of the primary leukaemia.[35] Whether a $CD34^{+}CD19^{-}$ progenitor/stem cell also lies at the basis of infant MLL carrying other types of *MLL* translocations remains to be confirmed.

PRENATAL ORIGIN OF INFANT ALL

It is believed that the first genetic event necessary for the development of childhood ALL frequently originates *in utero*, following additional postnatal mutations that lead to clinically overt leukemia. This seems particularly true for children ≤3 years of age as leukemic cells from these patients predominantly exhibit fetal-type DJ_H junctions of the complementarity determining region 3 (CDR3) of the immunoglobulin H chain that lack so called N regions, which are added during DJ_H recombination events later in fetal development[36]. Surprisingly, Fasching *et al* did find N regions to be present in infant ALLs specifically carrying t(4:11) translocations, suggesting that the initial transforming events took place at a later developmental stage when compared to children who developed leukemia between 1 and 3 years of age.[37] The prenatal origin for t(4:11) positive infant leukemias, however, has been well established by studies identifying unique clonotypic MLL-AF4 fusion sequences in neonatal blood spots from children developing ALL within the first two years of life.[38] Moreover, detection of identical clonal non-constitutive *MLL* translocations in the leukemic cells from several identical twins, strongly suggests that malignant cells with acquired *MLL* rearrangements of one twin were transferred to the other fetus by intra-placental metastasis.[39-42] Thus, with infant MLL indisputably originating *in utero*, but yet diplaying N-region positive DJ_H junctions strongly suggests an exquisitely short latency period for these leukemias to develop into overt disease. This implies that upon the development of the first genetic hit (presumably the *MLL* translocation) only limited mutagenic events are further required. Alternatively, the initial genetic event in infant MLL may create genetically highly instable genomes that are particularly susceptible to additional genetic alterations, additionally required to fully develop this type of leukemia.

ETIOLOGY AND RISK FACTORS

As with most cancers, the cause of infant ALL largely remains unknown. However, several factors have been noted that increase the risk to develop infant ALL. As the initiating event of most (if not all) infant leukemias occur *in utero*, factors that promote

malignant transformation in these children most likely strike shortly before or during pregnancy. For example, both maternal alcohol consumption during pregnancy and paternal smoking one month prior to pregnancy have been shown to be associated with increased risk of infant ALL.[43] Moreover, there are indications that maternal exposure during pregnancy to low-dose radiation from the Chernobyl accident has been responsible for an excess of infant ALL cases in contaminated areas (reviewed by Moysich *et al*[44]). Furthermore, several studies in children <4 years of age suggested that a high birth weight and a maternal history of fetal loss are associated with increased risk of developing ALL.[45-47] However, Ross *et al* showed that only high birth weight, complied as a significant risk factor for developing ALL within the first year of life.[48]

Rearrangements involving the *MLL* gene frequently arise in therapy-related secondary leukemias in older children exposed to drugs that function as topoisomerase II inhibitors like epipodophyllotoxins (such as etoposide) and anthracyclines. At first intensive treatment with topo-II inhibitors appeared to predominantly result in the development *MLL* rearranged AML[49,50], however, increasing numbers of cases of therapy-related *MLL* rearranged ALL are emerging (reviewed by Anderson *et al*[51]). These findings led to the hypothesis that transplacental exposure to topo-II inhibitors may be involved in the etiology of infant MLL.[52] This hypothesis is strongly supported by the finding that both primary infant MLL and therapy-related secondary acute leukemias characterized by translocation t(4;11) show *MLL* breakpoints that are similarly distributed within the *MLL* breakpoint cluster region.[53] Several dietary bioflavonoids like quercetin (found in certain fruits and vegetables) and genistein (soy) are known topo-II inhibitors, and are capable of crossing the placenta[54], and were shown to induce *MLL* cleavage *in vitro*.[55] These data led to the postulation that maternal consumption of dietary bioflavonoids may potentially lead to *MLL* translocations *in utero* and contribute to the development of infant MLL. However, as yet only an increased risk of infant AML, but not of ALL, has actually been linked to maternal consumption of food containing topo-II inhibitors.[56] Moreover, recently reported contradicting results demonstrate that maternal consumption of fresh vegetables and fruits during pregnancy was associated with a decreased instead of increased, risk of infant MLL.[57]

A common structural feature shared by many topo-II inhibitors (including flavonoids) is a quinone moiety. Metabolites generated as by-products of the metabolism of quinone-containing compounds within the fetal liver, thus include quinones[58], which have been shown to cleave both the *MLL* gene as well as its fusion partner *AF4* at topo-II cleavage sites.[59] Thus, apart from intact topo-II inhibitors, their quinone-containing metabolites may also be involved in the development of infant MLL. Nevertheless, the window of opportunity for quinones to induce DNA cleavage usually is rather small as they are normally detoxified by NAD(P)H:quinone oxidoreductase 1 (NQO1). However, the *NQO1* gene, is subject to a C→T polymorphism at nucleotide 609 in exon 6

which results in an amino-acid change (Pro187Ser) and gives rise to a NQO1 protein with significantly decreased enzymatic activity.[60,61] Interestingly, the occurrence of alleles generating low activity variants of NQO1 have been associated with increased risk of infant MLL[62], but not with ALL without *MLL* rearrangements in older children.[63] Taken together this suggests that elongated exposure to quinones may specifically induces *MLL* rearranged leukemias. More recently, however, a similar study has been reported in which this polymorphism appeared to be associated with infant ALL without *MLL* rearrangements, but not with infant MLL.[64]

PROGNOSTIC FACTORS IN INFANT ALL

In infant ALL, many prognostic factors have been described. All of these factors, summarized in Table 2, have been shown to confer a poor outcome mostly in univariate analyses, but also are closely related to each other.[19,20,65] In multivariate analysis including these factors, the presence of *MLL* rearrangements often remains the only independent factor predicting a poor outcome.[19,21,65] The event-free survival (EFS) for *MLL* rearranged infant cases (5–34%) is significantly worse than for their *MLL* germ line counterparts (42-92%) (Table 2). Some studies reported that only t(4;11) positive infant ALL patients experience a poor prognosis, whereas patients carrying other types of *MLL* rearrangements fare equally well as *MLL* germ line cases.[3,65] However, in a large cohort of infant MLL cases all types of *MLL* rearrangements were associated with a poor prognosis at this age[66], which also appears to be true for the majority of studies identifying *MLL* rearrangements as a prognostic factor (Table 2).

Although CD10 negativity (or a so-called pro-B phenotype), age less than 6 months, high white blood cell (WBC) counts, and central nervous system (CNS) involvement are strongly associated with the presence of *MLL* rearrangements, several studies also identified these clinical features as independent predictors of a poor prognosis (Table 2). Nevertheless, it can be concluded that the presence of *MLL* rearrangements and young age are the strongest independent prognostic factors. This is confirmed by preliminary results from the very large collaborative infant ALL study Interfant-99, which is currently in progress (coordinated by Dr. R. Pieters). Finally, Dordelmann *et al* demonstrated that a poor *in vivo* response to prednisone appeared to be a strong adverse prognostic factor, even among *MLL* rearranged cases.[6]

Table 2. Prognostic factors in infant ALL (<1 year of age).

Prognostic factor	Unfavorable / favorable feature	Sample size	Outcome (≥3yr-EFS)	Type of analysis	References
MLL gene rearrangements	Present / absent	n=96	19% (n=78) vs. 46% (n=18)	Univariate	Rubnitz, *et al* (1994)[131]
		n=40	28% (n=29) vs. 100% (n=11)[§]	Univariate	Hilden, *et al* (1995)[132]
		n=37	5% (n=27) vs. 89% (n=10)[¶]	Univariate	Taki, *et al* (1996)[20]
		n=59	28% (n=29) vs. 42-56% (n=30)	Univariate	Dordelmann, *et al* (1999)[6]
		n=55	34% (n=42) vs. 92% (n=13)	Univariate	Isoyama, *et al* (2002)[133]
		n=28	13% (n=19) vs. 67% (n=9)	Multivariate	Pui, *et al* (1994)[134]
		n=38[#]	9% (n=24) vs. 57% (n=14)	Multivariate	Cimino, *et al* (1995)[19]
		n=27	6% (n=18) vs. 56% (n=9)	Multivariate	Pui, *et al* (1996)[21]
		n=56	5% (n=21) vs. 47% (n=28)[*]	Multivariate[*]	Heerema, *et al* (1999)[65]
		n=82	3% (n=33) vs. 50% (n=49)[*]	Multivaraite[*]	Reaman, *et al* (1999)[3]
Age	<6 / ≥6 months	n=30	15% (n=13) vs. 44% (n=17)	Univariate	Pui, *et al* (1994)[134]
		n=29	8% (n=13) vs. 42% (n=16)	Univariate	Pui, *et al* (1996)[21]
		n=82	18% (n=26) vs. 57% (n=56)	Univariate[†]	Frankel, *et al* (1997)[2]
		n=23	30% (n=9) vs. 71% (n=14)	Univariate	Silverman, *et al* (1997)[5]
		n=56	Not specified	Univariate	Heerema, *et al* (1999)[65]
		n=44[**]	28% (n=25) vs. 63% (n=19)	Univariate	Kosaka, *et al* (2004)[135]
		n=88	8% (n=38) vs. 44% (n=50)	Multivariate	Chessells, *et al* (1994)[136]
		n=106	32% (n=50) vs. 56% (n=56)	Multivariate	Dordelmann, *et al* (1999)[6]
		n=222	13% vs. 26-49%	Multivariate[‡]	Reaman, *et al* (1999)[3]
WBC count	≥100 / <100 x10^9/L	n=24	Not specified	Univariate[‖]	Ishii, *et al* (1991)[137]
		n=88	24% (n=45) vs. 33% (n=43)	Univariate[‖]	Chessells, *et al* (1994)[136]
		n=23	36% (n=14) vs. 86% (n=9)	Univariate	Silverman, *et al* (1997)[5]
		n=106	37% (n=60) vs. 52% (n=46)	Univariate	Dordelmann, *et al* (1999)[6]
		n=222	Not specified	Multivariate[‖]	Reaman, *et al* (1999)[3]
Immuno-phenotype	CD10 negative / positive	n=106[#]	27% (n=37) vs. 50% (n=69)	Univariate	Basso, *et al* (1992)[26]
		n=71	21% (n=47) vs. 54% (n=24)	Univariate	Chessells, *et al* (1994)[136]
		n=28	28% (n=16) vs. 57% (n=12)	Univariate	Ferster, *et al* (1994)[7]
		n=37	10% (n=27) vs. 86% (n=10)[¶]	Univariate	Taki, *et al* (1996)[20]
		n=21	40% (n=10) vs. 73% (n=11)	Univariate	Silverman, *et al*., (1997)[5]
		n=104	35% (n=62) vs. 55% (n=42)	Univariate	Dordelmann *et al* (1999)[6]
		n=45	30% (n=28) vs. 56% (n=17)	Univariate	Heerema, *et al* (1999)[65]
		n=152	27% (n=98) vs. 56% (n=54)	Multivariate	Reaman, *et al* (1999)[3]
	Myeloid antigen co-expression present / absent	n=43	17% (n=14) vs. 39% (n=29)	Univariate	Basso, *et al* (1992)[26]
		n=22	10% (n=10) vs. 67% (n=12)	Univariate	Pui, *et al* (1994)[134]
CNS involvement	Present / absent	n=41[**]	10% (n=10) vs. 57% (n=31)	Univariate	Kosaka, *et al* (2004)[135]
		n=106	19% (n=45) vs. 51% (n=81)	Multivariate	Dordelmann, *et al* (1999)[6]
In vivo prednisone response	Poor response / good response[≍]	n=105	15% (n=27) vs. 53% (n=78)	Multivariate	Dordelmann, *et al* (1999)[6]

[§]2-yr EFS. [¶]Overall survival (OS) instead of event free survival (EFS). [#]Patients aged between 0 and 18 months. [*]t(4;11) cases only. [†]Age less than 9 months. [‡]Age less than 3 months. [‖]White blood cell count ≥50 x10^9/L. [**]*MLL* rearranged cases only. [≍]Poor prednisone response is defined as the presence of ≥1000 blasts/μL after a 7-day prednisone window (including one intrathecal dose of methotrexate on day one).

CELLULAR DRUG RESISTANCE AND WHY CURRENT THERAPY FAILS

Cellular drug resistance seems to significantly contribute to the dismal prognosis of infant ALL. About 30% of infant ALL patients show a poor *in vivo* response to prednisone[6], compared with ~10% of all children with ALL.[67] In addition, we showed that, compared with cells from older children diagnosed with ALL, leukemic cells from infant ALL patients are highly resistant to glucocorticoids (prednisone and dexamethasone) and L-asparaginase *in vitro*.[68,69] As the *in vitro* and *in vivo* response to prednisone is highly predictive for clinical outcome in childhood ALL in general[6,70,71], the poor prognosis for infant ALL may to a large extent be associated with cellular resistance to glucocorticoids. Interestingly, regardless of age, childhood pro-B ALL, which is the most commonly observed immunophenotype in infants with ALL (particularly in patients carrying translocation involving *MLL*), shows a similar drug resistance profile as observed for infant ALL.[69] Given that glucocorticoids generally fail to induce leukemic cell death in AML cells[72,73], the unfavorable response to these drugs, especially in pro-B/infant MLL may well be an epiphenomenon related to the differentiation stage of immature *MLL* rearranged pro-B ALL cells, that still display myeloid characteristics like the expression of myeloid-associated antigens (e.g. CD15 and CDw65).

Although infant MLL cells are relatively resistant to glucocorticoids when compared with other ALL subtypes, glucocorticoids should not be excluded from infant MLL treatment protocols, as the vast majority of leukemic cells are eliminated by prednisone[6], and complete remission (CR) is achieved in virtually all cases (~95%) on glucocorticoid-containing induction therapies.[2,3] The duration of these CRs, however, usually is rather short and typically occur within the first year following diagnosis. This suggests that small numbers of surviving cells rapidly give rise to a re-emerging leukemia. Surviving therapy, these cells presumably are even more resistant than the bulk of the leukemic cell population that was eliminated, simply as a result of selection. Reflecting this latter point, most patients who experienced a relapse do not survive their disease.[3] As immature (leukemic) stem cells usually are not prone (yet) to undergo apoptosis[74], the self-renewing $CD34^{+}CD19^{-}$ leukemic stem cells from which t(4;11) positive infant ALL seems to originate[34], most likely are some of the few residual cells surviving initial therapy. Therefore, adequate treatment for infant MLL may not only require more efficient targeting of the bulk of the leukemic population, but may also demand targeting of the self-renewing leukemic stem cell.

THE CONUNDRUM OF CELLULAR DRUG RESISTANCE

An essential step of glucocorticoid-induced leukemic cell death, is binding to the intracellular glucocorticoid receptor (GR).[75] Possibly, the presence of either mutations or polymorphisms within the *GR* gene may lead to impaired binding of the glucocorticoid-GR complex, and as a result, to increased resistance to these drugs. To test this hypothesis, we recently screened the entire *GR* gene for the presence of genetic alterations in a large group of pediatric ALL patients, including a number of infant ALL samples. Several polymorphisms but not mutations were found in the coding region of the *GR* gene of childhood ALL cells. Correlating these data to both *in vitro* and *in vivo* glucocorticoid cytotoxicity, however, showed that these genetic variations did not contribute to glucocorticoid resistance.[76]

L-asparaginase exerts its cytotoxic effects by depleting cells of asparagine and glutamine. It is believed that ALL cells are specifically sensitive to L-asparaginase due to their impaired capacity to synthesize asparagine as a result of reduced expression of asparagine synthetase (AS).[77,78] However, the hypothesis that resistance of infant ALL cells towards L-asparaginase may be explained by elevated expression levels of *AS*, could not be confirmed (R.W. Stam, unpublished observations).

Finally, the chemo-resistant character of infant MLL cells may suggest the involvement of multidrug resistance (MDR) proteins, which are specialized membrane transporters capable of trafficking multiple drugs out of the cell. Nevertheless, we recently showed that drug resistance in infant ALL is not likely a result of increased drug efflux mediated by MDR pumps, such as P-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1), lung resistance-related protein/major vault protein (LRP/MVP), and breast cancer resistance protein (BCRP), as the expression of the genes encoding these proteins is not elevated in infant ALL cells when compared with ALL cells from older children.[79]

Although highly informative, studies like these exclude only limited numbers of possible explanations for drug resistance in infant ALL at a time. As described later in this review, the rapidly advances in gene expression profiling techniques nowadays available may notably accelerate our progress in understanding cellular drug resistance in infant ALL.

NUCLEOSIDE ANALOGUE SENSITIVITY AND THERAPEUTIC POSSIBILITIES FOR INFANT ALL

Although relatively resistant to several chemotherapeutic drugs, we showed that infant ALL cells are more sensitive to the cytidine analogue cytosine arabinoside (Ara-C)

when compared with cells from older children with ALL.[68,69] Sensitivity to Ara-C in infant ALL appeared not to be associated with rearrangements of the *MLL* gene, as both *MLL* rearranged and *MLL* germ line infant ALL cases appeared equally sensitive to this drug *in vitro* (Stam, *et al* manuscript submitted). The remarkable sensitivity to Ara-C seems most likely to be due to elevated expression of the human equilibrative nucleoside transporter 1 (hENT1)[80], on which Ara-C is mainly dependent to permeate the cell membrane.[81-83] However, at high-dose Ara-C regimens generating high extracellular drug concentrations, Ara-C also enters the cell by passive diffusion and membrane transport via ENT1 is circumvented. Thus, infant ALL patients may benefit from their elevated *ENT1* expression when treated with low to moderate dosages of Ara-C, whereas this advantage presumably is lost when treated with high-dose Ara-C regimens. Nevertheless, improved outcomes have been reported for infant ALL patients treated with protocols in which high-dose Ara-C had been implemented during the consolidation phase.[3,5] Moreover, improved outcome for adult pro-B ALL cases (both *MLL* rearranged and germ line) was noted with intensified post-remission therapy including high-dose Ara-C.[84] In 1999 the collaborative Interfant-99 treatment protocol for infant ALL was initiated that included the intensive use of both low and high-dose Ara-C throughout the duration of the treatment.

In addition to Ara-C, infant ALL cells also appeared to be highly sensitive to the adenosine analogue 2-CdA (2-chlorodeoxyadenosine or cladribine).[69] Whether 2-CdA sensitivity in infant ALL can also be attributed to increased *ENT1* expression remains uncertain. Wright *et al* showed that 2-CdA does not seem to enter pediatric ALL cells via the same membrane nucleoside transport system responsible for cellular Ara-C influx[85]. However, the number of ALL samples used in that study was rather small. In contrast, we recently observed a significant correlation between *ENT1* expression and sensitivity to 2-CdA in a large cohort of childhood AML patients.[86] Interestingly, in several studies synergistic effects between Ara-C and 2-CdA have been observed *in vitro*[87-89], and the addition of 2-CdA to Ara-C containing regimens have been shown to increase the complete remission rates in AML.[90,91] Taken together these observations support that regimens combining the use of Ara-C and 2-CdA may potentially be beneficial for infant ALL patients. Moreover, given the apparent sensitivity of infant ALL cells to nucleoside analogues, the use of newly developed nucleoside analogous like for example clofarabine[92,93] and troxacitabine (troxatyl)[94,95], may be interesting candidate drugs for further testing on infant MLL cells.

Another specific class of nucleoside analogue drugs that may additionally be effective against infant MLL cells are DNA demethylating cytidine analogues, such as 5-azacytidine, 5-aza-2'-deoxycytidine (decitabine), or the recently identified agent zebularine.[96] Gutierrez *et al* showed that among several pediatric ALL subtypes, *MLL* rearranged cases had the highest methylation index (i.e. number methylated genes/

number of genes studied).[97] Thus, MLL seems to be characterized by aberrant DNA hypermethylation. In concordance with this, we recently observed that the tumor suppressor gene *FHIT* was silenced by 5'CpG island methylation in 100% of the infant MLL cases tested, whereas silencing of this gene in older children with ALL carrying germ line *MLL* genes was observed in only ~50% of the cases.[98] Furthermore, we observed that ectopic expression of *FHIT* in the MLL cell line RS4;11, which lacks endogenous *FHIT* expression, induced leukemic cell death. Likewise, exposing MLL cells to the demethylating agent decitabine resulted in re-expression of FHIT protein expression and induced apoptosis. Therefore, inhibition of DNA methylation may be an effective therapeutic strategy in the treatment of infant MLL, especially since we recently found that decitabine (like other cytosine analogues) depend on ENT1 to cross the cell membrane,[86] which is highly expressed in infant ALL cells.[80]

Nevertheless, as combinations of multiple chemotherapeutic drugs rather than single (types of) agents are required to cure acute leukemias, extending the drug repertoire against infant MLL with several nucleoside analogues will probably not be sufficient to significantly improve the prognosis. Thus, in addition to exploring the use of effective nucleoside analogues, other innovative treatment strategies are needed that either overcome resistance to conventional drugs or which involve alternative novel agents that more effectively target infant MLL cells.

NOVEL THERAPEUTIC TARGETS

The rapidly advanced gene expression profiling technologies, nowadays allow comparisons of multiple patient groups for the expression of vast numbers of genes, by approach covering the entire genome. Recently, we demonstrated how such a gene expression profiling study can be a suitable approach in gaining new insights in drug resistance mechanisms at a genetic level. Comparing gene expression patterns in childhood ALL patients either resistant or sensitive to prednisone, vincristine, L-asparaginase, and daunorubicin *in vitro*, we found 124 differentially expressed genes to be related to resistance to one of these drugs.[99] Moreover, this gene expression signature associated with drug resistance appeared to be highly predictive for clinical outcome. Interestingly, only three of the 124 differentially expressed genes had been associated with drug resistance before, indicating that the mechanisms underlying resistance to these drugs are complex and largely unknown. Validation studies are now needed to determine the exact mechanisms that are involved, and whether these genes are causally involved in resistance. Some of these genes may represent universal determinants of drug resistance that apply to all types of leukemia, including infant MLL. For example, one of the genes that appeared to be over-expressed in prednisone-resistant ALL cells was *MCL-1*,

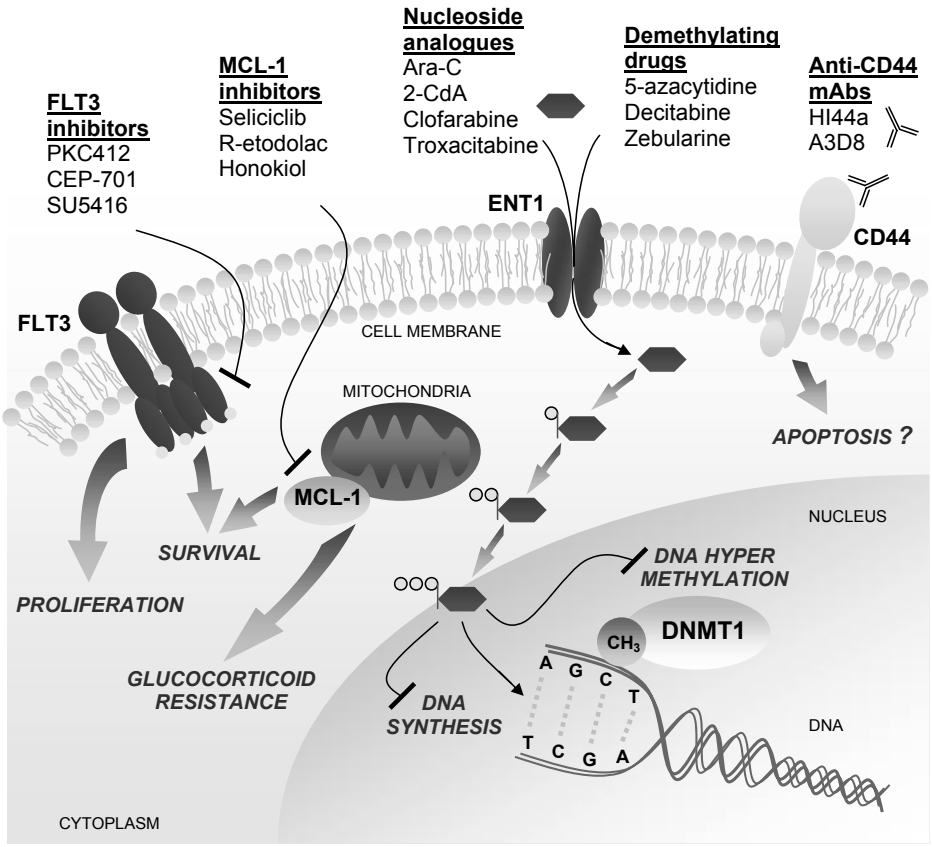

Figure 1. Schematic and simplified representation of putative therapeutic strategies against infant ALL. FLT3 activation involves receptor dimerization and phosphorylation. Constitutively activated FLT3 promotes proliferation and leukemic cell survival. Additionally contributing to apoptotic resistance are elevated expression levels of MCL-1, which seems to particularly mediate resistance to glucocorticoid induced cell death. Nucleotides (depicted as hexagons) and their analogues are hydrophilic molecules and therefore require specialized membrane transport proteins (e.g. ENT1) to permeate leukemic cells. Inside the cell, nucleosides are being phosphorylated to form nucleotide triphosphates, and become incorporated into the DNA. Incorporated nucleoside analogue drugs such as Ara-C and 2-CdA induce apoptosis by blocking DNA synthesis. Incorporated demethylating cytidine analogues inhibit DNA (hyper)methylation by forming covalent complexes with DNA methyltransferases (like DNMT1), depleting the cell of functional DNMTs.

an anti-apoptotic member of the BCL-2 family.[99] Interestingly, we observed increased *MCL-1* expression in infant MLL samples when compared with samples from older children with ALL, and found the expression of this gene to correlate with resistance to prednisone both in infant and non-infant ALL (Stam, *et al* unpublished data). MCL-1 plays an important role in the survival of B-cell chronic lymphoblastic leukaemia (B-CLL)[100], and multiple myeloma (MM) cells[101], in which *MCL-1* also is abundantly expressed. Recently, honokiol (a natural phenolic compound extracted from the root and stem bark of several *Magnolia* species) was found to induce leukemic cell death

in primary B-CLL cell, by down-regulating MCL-1.[102] Likewise, two synthetic MCL-1 inhibitors, i.e. Seliciclib (CYC202 or R-roscovitine) and the R-etodolac (SDX-101) were shown to induce apoptosis in MM cells by directly targeting MCL-1.[103,104] Interestingly, sub-cytotoxic doses R-etodolac sensitized MM cells to dexamethasone-induced cell death. Therefore, glucocorticoid resistance in infant MLL cells might be overcome by compounds like Seliciclib and R-etodolac, or may directly induce leukemic cell death. Hence, inhibition of MCL-1 may be another therapeutic possibility for infant MLL. Importantly, MCL-1 also plays an important role in the development of B and T lymphocytes[105] as wells as in the survival of hematopoietic stem cells.[105,106] Therefore, targeting MCL-1 in infant MLL cells may also induce leukemic cell death of $CD34^{+}CD19^{-}$ leukemic stem cells.

On the other hand, given the unique biological and clinical features associated with infant MLL, it seems likely that next to general drug resistance mechanisms, distinct mechanisms are also involved that are more specific for this type of leukemia. Hence, in addition to our micro-array studies in older children with ALL[99], additional studies are being conducted on infant MLL patients in order to gain insights in the specific genes involved in drug resistance.

Like the comparison of gene expression profiles from drug sensitive and resistant patients may lead to the identification of genes involved in drug resistance, comparison of leukemia subtypes may identify genes that are uniquely expressed within certain types of leukemia, allowing the development of subtype-specific therapy. Several studies have demonstrated that based on their gene expression profiles, *MLL* rearranged ALL specifies a unique type of leukemia displaying a gene expression pattern that is clearly distinguishable from other genetic subtypes of ALL.[23,24,107,108] Soon after this finding, this MLL-specific gene expression signature proved to be of great value for the discovery of novel therapeutic targets.

For example, *FLT3*, the gene encoding Fms-like tyrosine kinase 3, appeared to be one of the genes most consistently highly expressed in patients with MLL.[23] FLT3 is important in early B-lineage development[109] and as such is most abundantly expressed in immature B-cells[110], which may explain the high expression of this gene in MLL cells, which typically display immature pro-B phenotypes. Normally FLT3 becomes activated upon binding of the hematopoietic growth factor FLT3 ligand (FLT3L). However, in AML the *FLT3* gene is frequently subjected to mutations that constitutively activate this receptor in a ligand-independent manner, providing leukemic cells with a growth advantage and transforming capacity (reviewed in ref.111). As such, constitutively activated FLT3 became a promising therapeutic target in AML. Several small molecule inhibitors (e.g. CEP-701, PKC412, and SU5416) efficiently inactivate FLT3 and induce leukemic cell death *in vitro* in AML cells depending on constitutive FLT3 signaling.[112-114] This finding prompted the initiation of several phase I/II clinical trials to determine

the efficacy of these inhibitors in refractory AML patients, and so far the results are promising.[115-117] Interestingly, constitutively activated FLT3 also occurs in MLL patients carrying activating mutations, and in MLL patients merely displaying high-level expression of wild-type *FLT3*.[118,119] We and others recently demonstrated that high-level wild-type *FLT3* expression in primary infant MLL samples is associated with activated FLT3 and cytotoxic responsiveness to FLT3 inhibitors.[119,120] These data show that FLT3 inhibition may represents a novel therapeutic strategy for infant MLL that urgently demands clinical testing.

Interestingly, in combination with Ara-C, some FLT3 inhibitors display synergistic cytotoxic effects in leukemic cells that are dependent on FLT3 activation.[121,122] This, together with the *in vitro* responsiveness of MLL cells to FLT3 inhibition, suggests that the addition of FLT3 inhibitors to Ara-C containing regimens may possibly improve treatment response for infant MLL. The sequence of administration, however, seems rather important. In order to achieve a synergistic effect, Ara-C and the FLT3 inhibitor CEP-701 should be administered simultaneously, or CEP-701 should be given directly following Ara-C. In contrast, pre-exposure to CEP-701 followed by Ara-C administration led to antagonistic effects.[121]

Noteworthy is the recent finding that FLT3 activating internal tandem duplications (ITDs) as observed in primary AML samples[123], are also present in the $CD34^+/CD39^-$ leukemic stem cell fraction of these samples.[124] Moreover, exposing these FLT3/ITD positive stem cells to the FLT3 inhibitor CEP701, inhibited engraftment of these cells.[124] These data indicate that FLT3 inhibition in AML patients carrying this type of activating *FLT3* mutation may be highly effective, as the leukemic stem cell is targeted as well. If constitutively activated FLT3 as a consequence of over-expression can be demonstrated in the $CD34^+CD19^-$ leukemic stem cell fraction in primary infant MLL samples[34], FLT3 inhibition may also effectively target these leukemic stem cells.

Another example of a potential therapeutic target for infant MLL identified by gene expression profiling may be CD44. CD44 is a cell surface antigen that is strongly expressed on leukemic blasts from most AML patients.[125] Interestingly, several studies reported apoptosis-inducing effects of specific monoclonal antibodies (e.g. HI44a and A3D8) against CD44 on both AML cell lines and primary AML cells.[126-129] Gene expression profiling revealed that CD44 also is highly expressed on MLL cells as compared to other ALL subtypes.[23,130] This similarity to AML cells again stresses the biphenotypic character of infant MLL, and imply that antibody based treatment approaches targeting CD44 on infant MLL cells, may have therapeutic potential. The possible value of CD44, as well as of other candidate genes identified by gene expression profiling, as therapeutic targets for infant MLL are currently being studied in our laboratory.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Infant ALL is a rare malignancy that is characterized by an exceedingly high incidence (~80%) of *MLL* gene rearrangements, which is a strong predictor of an adverse outcome. Current therapies fail in >50% of the *MLL* rearranged infant ALL (MLL) cases, indicating that innovative therapeutic strategies are urgently needed to improve prognosis. It is, therefore, of utmost importance to unravel the unique molecular biological properties of this malignancy. As shown in this review, this already provides insights into what type(s) of drugs may be effective against infant MLL, and may provide a sense for why other classes of drugs are highly ineffective. Furthermore, genome-wide analysis has led to the identification of genes specifically expressed in infant MLL that may serve as therapeutic targets. Therefore, continued molecular studies designed to validate the potential of such targets, or to further gain insight in the biology of infant MLL, should ultimately lead to the development of effective treatment regimens for this aggressive type of leukemia.

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Chapter 3

Differential mRNA expression of Ara-C metabolizing enzymes explains Ara-C sensitivity in *MLL* gene rearranged infant acute lymphoblastic leukemia (ALL)

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ABSTRACT

Infant acute lymphoblastic leukemia (ALL) is characterized by a high incidence of *MLL* gene rearrangements, a poor outcome, and resistance to chemotherapeutic drugs. One exception is cytosine arabinoside (Ara-C), to which infant ALL cells are highly sensitive. To investigate the mechanism underlying Ara-C sensitivity in infants with ALL, mRNA levels of Ara-C metabolizing enzymes were measured in infants (n=18) and non-infants with ALL (n=24). In the present study, infant ALL cells were 3.3-fold more sensitive to Ara-C (p=0.007) and accumulated 2.3-fold more Ara-CTP (p=0.011) upon exposure to Ara-C, compared with older children with ALL. Real-time quantitative RT-PCR (TaqMan) analysis revealed that infants express 2-fold less of the Ara-C phosphorylating enzyme deoxycytidine kinase (*dCK*) mRNA (p=0.026) but 2.5-fold more mRNA of the equilibrative nucleoside transporter 1 (*hENT1*), responsible for Ara-C membrane transport (p=0.001). The mRNA expression of pyrimidine nucleotidase I (*PN-I*), cytidine deaminase (*CDA*) and deoxycytidylate deaminase (*dCMPD*) did not differ significantly between both groups. *hENT1* mRNA expression inversely correlated with *in vitro* resistance to Ara-C (r_s=-0.58, p=0.006). The same differences concerning *dCK* and *hENT1* mRNA expression were observed between *MLL* gene rearranged (n=14) and germline *MLL* cases (n=25). An oligonucleotide microarray screen (Affymetrix) comparing *MLL* gene rearranged ALL with non-rearranged ALL patients, showed a 1.9-fold lower *dCK* (p=0.001) and a 2.7-fold higher *hENT1* (p=0.046) mRNA expression in *MLL* gene rearranged ALL patients. We conclude that an elevated expression of hENT1, which transports Ara-C across the cell membrane, contributes to Ara-C sensitivity in *MLL* gene rearranged infant ALL.

INTRODUCTION

Although the treatment of childhood acute lymphoblastic leukemia (ALL) has improved tremendously over the last few decades, for some subgroups of patients the prognosis still remains poor. Infants (i.e. children ≤12 months of age) form such a subgroup. Infant ALL is characterized by a high incidence of rearrangements of the Mixed Lineage Leukemia (*MLL*, *ALL-1* or *HRX*) gene on chromosome band 11q23. The frequency of these *MLL* gene rearrangements is possibly as high as 75% when detected with molecular techniques.[1] The most common *MLL* abnormalities found in infants with ALL are the translocations t(4;11) and t(11;19) occurring in approximately 70% and 15% of the *MLL* gene rearranged cases, respectively.[2,3,4,5] The immunophenotype of *MLL* gene rearranged infant ALL is usually that of an immature precursor B-lineage lacking CD10 expression and co-expressing myeloid associated antigens. Furthermore, infants with ALL have a poor prognosis compared with older children with ALL, with an event-free survival (EFS) of ~35%.[6] The most important reason for this poor prognosis is cellular drug resistance. Pieters *et al*[7] showed that leukemic cells from infants with ALL are *in vitro* significantly more resistant, especially to prednisone and L-asparaginase, than cells from older children with ALL. One exception, however, is cytosine arabinoside (Ara-C) to which infant ALL cells are highly sensitive.[7] These findings have led to the development of a new treatment protocol for infants with ALL i.e. the INTERFANT-99 protocol.

Ara-C is a deoxycytidine analogue that is phosphorylated into its active form Ara-CTP which competes with dCTP for incorporation into DNA. When incorporated, Ara-C blocks DNA synthesis and as a consequence the cell is subjected to programmed cell death (Figure 1). Nucleosides and their analogues are hydrophilic molecules and therefore require specialized membrane transport proteins to be transported into cells[8]. To permeate the cell membrane, Ara-C is mainly dependent on the human equilibrative nucleoside transporter 1 (hENT1).[9,10] Inside the cell, deoxycytidine kinase (dCK) phosphorylates Ara-C to form Ara-CMP, which is thought to be the rate-limiting activation step of Ara-C.[11] Subsequently, Ara-CMP is further phosphorylated into Ara-CDP by (deoxy)cytidylate kinase (UMP-CMPK) and finally into its active, cytotoxic form Ara-CTP by nucleotide diphosphate kinases (NDPKs). Pyrimidine nucleotidase I (PN-I) catalyzes the dephosphorylation of Ara-CMP[12], thereby opposing the action of dCK. Cytidine deaminase (CDA) and deoxycytidylate deaminase (dCMPD) convert Ara-C to Ara-U and Ara-CMP to Ara-UMP, respectively, by deaminating the cytosine base. Inactivation of Ara-C and Ara-CMP by these deaminating enzymes decreases the amount of Ara-CTP and thus the cytotoxic effects of Ara-C.[13,14]

The mechanism underlying the remarkable Ara-C sensitivity in infants with ALL is unknown. Hypothetically, increased activation of the pro-drug Ara-C to its active, cyto-

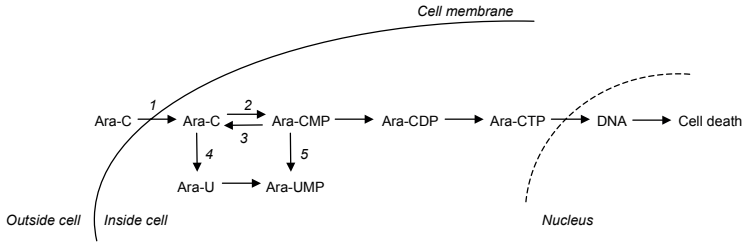

Figure 1. Cytosine arabinoside (Ara-C) metabolism within cells.
Ara-C enters the cell mainly via *(1)* equilibrative nucleoside transporter 1 (hENT1). Inside the cell, Ara-C is phosphorylated to Ara-CMP by *(2)* deoxycytidine kinase (dCK). Subsequently, Ara-CMP is phosphorylated into its active form Ara-CTP. Incorporation of Ara-CTP into the DNA during DNA synthesis leads to programmed cell death or apoptosis. Ara-CTP formation can, however, be obstructed. *(3)* Pyrimidine nucleotidase I (PN-I) inhibits Ara-CTP formation by opposing the action of dCK. *(4)* Cytidine deaminase (CDA) and *(5)* deoxycytidylate deaminase (dCMPD) convert Ara-C to Ara-U and Ara-CMP to Ara-UMP respectively, thereby decreasing the amount of Ara-CTP that can be formed.

toxic form Ara-CTP caused by aberrant expression of the above described enzymes may be involved. Accordingly we determined the mRNA levels of these Ara-C metabolizing enzymes in a group of 18 infants and 24 children older than 12 months of age diagnosed with ALL, using real-time quantitative RT-PCR (Taqman) analysis. In addition we used an oligonucleotide microarray screen[15] to compare the expression levels of these enzymes in *MLL* gene rearranged ALL patients and patients with conventional ALL.

MATERIALS ANS METHODS

Patient samples

Bone marrow and/or peripheral blood samples from untreated infants (i.e. children ≤12 months of age) initially diagnosed with ALL were collected from the University hospital Rotterdam / Sophia children's hospital and other hospitals participating in the INTERFANT-99 treatment protocol. Samples from initially diagnosed ALL patients older than 12 months of age were obtained from the German COALL study group (Prof. Dr. G. E. Janka-Schaub, Hamburg, Germany). Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/ml ; Nycomed Pharma, Oslo, Norway), centrifuged at 480 *g* for 15 minutes at room temperature. The collected mononuclear cells were washed twice and kept in culture medium consisting of RPMI 1640 medium (Dutch modification without L-glutamine ; Gibco BRL, Life Technologies), 20% fetal calf serum (FCS ; Integro, Zaandam , The Netherlands), 2 mM L-glutamine (Gibco BRL, Life Technologies) 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (ITS media supplement ; Sigma, St Louis MO, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.125 μg/ml

fungizone (Gibco BRL, Life Technologies) and 0.2 mg/ml gentamycin (Gibco BRL, Life Technologies). Contaminating non-leukemic cells were removed by immunomagnetic beads as described by Kaspers *et al.*[16] All samples contained ≥90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. For RNA extraction, a minimum of 5 x10^6 cells were lysed in TRIzol reagent (Gibco BRL, Life Technologies) and stored at -80 °C until extraction.

In vitro Ara-C cytotoxicity assay

In vitro Ara-C cytotoxicity was determined using the MTT assay as described by Pieters *et al.*[17] Briefly, 100 µl aliquots of cell suspension (~1.6 x10^5 cells) were cultured in round-bottomed 96-well microtitre plates (Greiner bio-one) in the presence of six different concentrations of Ara-C (Cytosar, Pharmacia & Upjohn BV, Woerden, The Netherlands) ranging from 0.009 – 10 µg/ml, in duplicate. Control cells were cultured in eight wells without Ara-C. 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 Aldrich, Zwijndrecht, The Netherlands) was added and the plates were incubated for an additional six hours under the same conditions. During this final 6-hour incubation, the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals by viable cells only. The formazan crystals were dissolved by adding 100 µl acidified isopropanol (0.04 N HCl-isopropyl alcohol) and the optical density (OD), which is linearly related to the number of viable cells,[18] was measured spectrophotometrically at 562 nm. After subtraction of the blank values, the leukemic cell survival (LCS) was calculated by the equation :

$$LCS = (OD_{Day\ 4}\ \text{treated well} / \text{mean}\ OD_{Day\ 4}\ \text{control wells}) \times 100\%$$

Drug sensitivity was assessed by the LC_{50}, the drug concentration lethal to 50% of the cells. Evaluable assay results were obtained when a minimum of 70% leukemic cells was present in the control wells after 4 days of incubation and when the control optical density (OD) was ≥ 0.050.[17]

Ara-CTP accumulation

Leukemic cells from patients, prepared as described above, were incubated at 37°C for 24 hours in the presence of 1 µM (0.25 µg/ml) of Ara-C. After exposure to Ara-C, cells were washed in drug-free medium, centrifuged for 3 minutes at 5000 rpm and rapidly frozen and stored at -80 °C. Ara-CTP was extracted as described by Noordhuis *et al.*[19] Cell pellets were resuspended in 150 µl ice-cold phosphate buffered saline (pH 7.4) and subsequently 50 µL of 40% ice-cold trichloroacetic acid (TCA) (w/v) was added.

The suspension was kept on ice for 20 minutes. After centrifugation for 5 minutes at 13000 rpm and 4°C, the supernatant was removed and neutralized by adding 400 µl of tri-octylamine/1,1,2-tri-chloro-trifluorethane (1/4, v/v). After further centrifugation for 1 minute, the upper layer (the nucleotide extract) was collected and stored at -20°C. Nucleotides were separated using anion-exchange high-performance liquid chromatography (HPLC)[19] on a Partisphere Sax column (Whatman; i.d. 4.6 mm, length 12.5 cm, particle size 5 µm). A 1000S diode-array detector was set at 280 and 254 nm (Applied Biosystems, Foster City, CA, USA) and a Chromeleon V 4.30 data acquisition system (Dionex, Breda, the Netherlands) was used for quantitation of the peaks. Elution was performed isocratically with 0.25 M KH_2PO_4 containing 0.5 M KCl (pH 4.5) at a flow of 1.5 ml/min. The retention time of Ara-CTP was 5.4 minutes.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from a minimum of 5 x10^6 cells using TRIzol reagent (Gibco BRL, Life Technologies) according to the manufacturer's protocol, except for minor modifications. An additional phenol-chloroform extraction was performed and the isopropanol precipitation at –20°C was facilitated by adding 1 µL (20 µg/ml) glycogen (Roche, Almere, The Netherlands). After precipitation with isopropanol, RNA pellets were dissolved in 20 µl RNAse-free TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0). The RNA was quantitated spectrophotometrically. Following a denaturation step of 5 min at 70°C, 1 µg of RNA was reverse transcribed to single stranded cDNA using a mix of random hexamers (2.5 µM), and oligo dT primers (20 nM). The RT reaction was performed in a total volume of 25 µl containing 0.2 mM of each dNTP (Amersham Pharmacia Biotech, Piscataway NJ, USA), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison Wisconsin, USA) and 25 U RNAsin (Promega, Madison Wisconsin, USA), at 37°C for 30 minutes, 42°C for 15 minutes and 94°C for 5 minutes. The obtained cDNA was diluted to a final concentration of 8 ng/µl. Samples were stored at –80°C.

Quantitative real-time PCR (Taqman technology)

The mRNA expression levels of *dCK*, *PN-I*, *CDA*, *dCMPD*, *hENT1* and an endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference, were quantified using real-time PCR analysis (Taqman chemistry) on an ABI Prism 7700 sequence detection system (PE Applied Biosystems). Amplification of specific PCR products was detected using dual-fluorescent non-extendable probes labeled with 6-carboxyfluorescein (FAM) at the 5'-end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end. All primers and probe combinations (Table 1) were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO, USA) and purchased from Eurogentec (Seraing, Belgium). All primers had a melting

Table 1. Probe and primer combinations used for quantitative real-time PCR (Taqman).

Gene	Primer/probe	Sequence
dCK	forward	5′-TGC AGG GAA GTC AAC ATT-3′
	reverse	5′-TCC CAC CAT TTT TCT GAG-3′
	probe	5′-(FAM)-TAA ACA ATT GTG TGA AGA TTG GGA AG-(TAMRA)-3′
CDA	forward	5′-GGA GGC CAA GAA GTC AG-3′
	reverse	5′-GAC GGC CTT CTG GAT AG-3′
	probe	5′-(FAM)-CAA CAT AGA AAA TGC CTG CTA CCC-(TAMRA)-3′
dCMPD	forward	5′-AAT GGG TGC AGT GAT GAC-3′
	reverse	5′-CTT AGC GCA TTC ATT ACA AG-3′
	probe	5′-(FAM)-ATC ATG AAC AAA AAT TCG ACC GAT-(TAMRA)-3′
PN-I	forward	5′-AAT CGG CGA TGT ACT AGA G-3′
	reverse	5′-CAT CTG CCA TTC TTA AGT CTC-3′
	probe	5′-(FAM)-ATG AAA CTG GGG TGC TCA AAG GA-(TAMRA)-3′
hENT1	forward	5′-TGT TTC CAG CCG TGA CT-3′
	reverse	5′-CAG GCC ACA TGA ATA CAG-3′
	probe	5′-(FAM)-CAG CAC CTG GGA ACG TTA CTT-(TAMRA)-3′
GAPDH	forward	5′-GTC GGA GTC AAC GGA TT-3′
	reverse	5′-AAG CTT CCC GTT CTC AG-3′
	probe	5′-(FAM)-TCA ACT ACA TGG TTT ACA TGT TCC AA-(TAMRA)-3′

temperature (T_m ; nearest neighbor method) of 65 ± 1°C. All internal probes had a T_m of 75 ± 1°C. All PCRs performed with comparable efficiencies of ≥95%. The quantitative real-time PCR was performed in a total reaction volume of 50 µL containing 1x Taqman buffer A (Applied Biosystems), 4 mM $MgCl_2$, 200 µM of each dNTP (Amersham Pharmacia Biotech, Piscataway NJ, USA), 300 nM forward and reverse primer, 50 nM dual-labeled fluorogenic internal probe, 1.25 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems) and 40 ng of cDNA (see above) from each patient as a template, in MicroAmp optical 96-well plates covered with MicroAmp optical caps (Applied Biosystems). Samples were heated for 10 minutes at 95°C and amplified for 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. A serial dilution of cDNA derived from a cell line RNA-pool (CEM, K562, and two EBV transformed lymphoblastic B-cell lines) in dH_2O was amplified in parallel as a control to verify amplification efficiency within each experiment. Since all PCRs performed with equal efficiencies, relative mRNA expression levels of *dCK*, *PN-I*, *CDA*, *dCMPD* and *hENT1* for each patient can directly be normalized for input RNA against the *GAPDH* expression of the patient. The relative mRNA expression levels of the target genes in each patient was calculated using the comparative cycle time (C_t) method.[20] Briefly, the target PCR C_t values, i.e. the cycle number at which emitted fluorescence exceeds 10 x the standard deviation (SD) of base-line emissions as measured from cycles 3 to 15, is normalized to the *GAPDH* PCR

C_t value by subtracting the *GAPDH* C_t value from the target PCR C_t value, which gives the ΔC_t value. From this ΔC_t value, the relative mRNA expression level to GAPDH for each target PCR can be calculated using the following equation :

$$\text{Relative mRNA expression} = 2^{-[Ct\ target - Ct\ GAPDH]} \times 100\%.$$

Oligonucleotide microarray screen (Affymetrix)

A detailed material and method section for the oligonucleotide microarray screen (Affymetrix) comparing *MLL* gene rearranged and non-rearranged ALL patients has been described elsewhere.[15] In addition, further details regarding patient samples and data analysis can be found at http://www.dfci.harvard.edu/korsmeyer/MLL.htm.

Statistics

Differences in the distribution of Ara-C LC_{50} values and mRNA expression between two groups were analyzed using the Mann-Whitney U test. Correlations between mRNA expression of Ara-C metabolizing enzymes and Ara-C LC_{50} values were calculated using the Spearman's rank correlation test. Statistical tests were performed at a two-tailed significance level of 0.05.

Table 2. Patient characteristics.

	Infants	Non-infants
Number (n)	18	24
Sex (male : female), (%)	67 : 33	67 : 33
Age, yrs. (median; P25 – P75[§])	0.45 (0.198 – 0.65)	5.5 (2.93 – 8.3)
Immunophenotype, (%)		
Pro-B ALL	56	0
Pre-B ALL	17	12
c-ALL	5	71
T-ALL	0	17
Unknown	22	0
MLL gene status, (%)		
MLL germline	11	100
MLL rearranged	78	0
Unknown	11	0

[§]P25 – P75 = 25th and 75th percentile (i.e. quartiles).

RESULTS

Leukemic cells from infants (n=18) and older children (n=24) newly diagnosed with ALL were used. Patient characteristics are listed in Table 2. The *in vitro* Ara-C cytotoxicity was successfully tested in 9 infants and 14 samples from older children with ALL. Assay failure was mostly due to a poor survival of control leukemic cells, i.e. <70 % survival of leukemic cells after 4 days of culture in the absence of Ara-C, or an optical density (OD) at 562 nm of <0.050. Leukemic cells from infants with ALL were significantly (p=0.007) more sensitive (3.3-fold) to Ara-C than cells from older children with ALL (Figure 2) with a median LC_{50} of 0.27 μg/ml Ara-C for the infant and 0.89 μg/ml Ara-C for the non-infant ALL samples. This is in concordance with results published before.[7] Furthermore, we determined Ara-CTP accumulation upon a 24 hour exposure to 1 μM (0.25 μg/ml) of Ara-C in 15 infant and 8 non-infant ALL samples. In two of the non-infant samples, a limited number of cells were available in which no Ara-CTP was detectable. These samples were considered to contain less than 30 pmol Ara-CTP per million leukemic cells. In leukemic cells from infants with ALL, 2.3-fold more Ara-CTP accumulated (p=0.011) (Figure 2).

Using quantitative real-time PCR (Taqman technology), the mRNA expression levels of *dCK*, *CDA*, *dCMPD*, *PN-I* and *hENT1* were measured in 18 infant ALL samples. In 23 non-infant samples the mRNA expression levels of *dCK*, *CDA* and *dCMPD* were determined. From 21 non-infants *PN-I* and *hENT1* mRNA expression was determined.

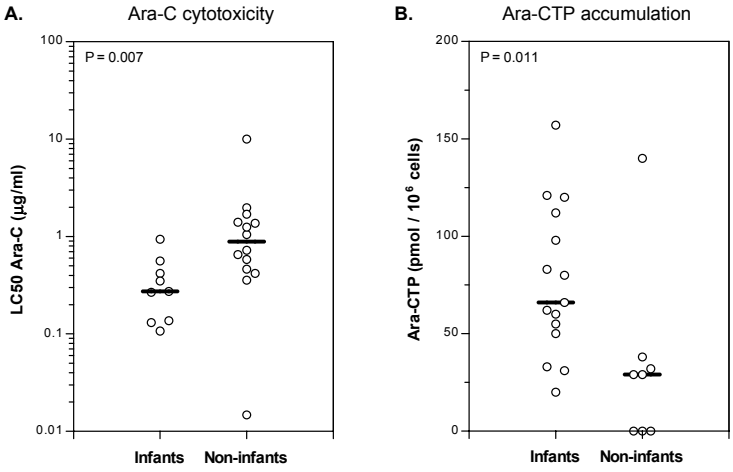

Figure 2. Ara-C cytotoxicity and Ara-CTP accumulation.
A. Ara-C sensitivity (LC_{50} Ara-C in μg/ml) of leukemic cells from infants (n=9) and non-infants (n=14) with newly diagnosed acute lymphoblastic leukemia (ALL). **B.** Ara-CTP accumulation (pmol/10^6 cells) in leukemic cells from infant (n=15) and non-infant (n=8) ALL patients upon *ex vivo* exposure to 1 μM of Ara-C for 24 h. The lines indicate the median values, circles (o) represent individual patients.

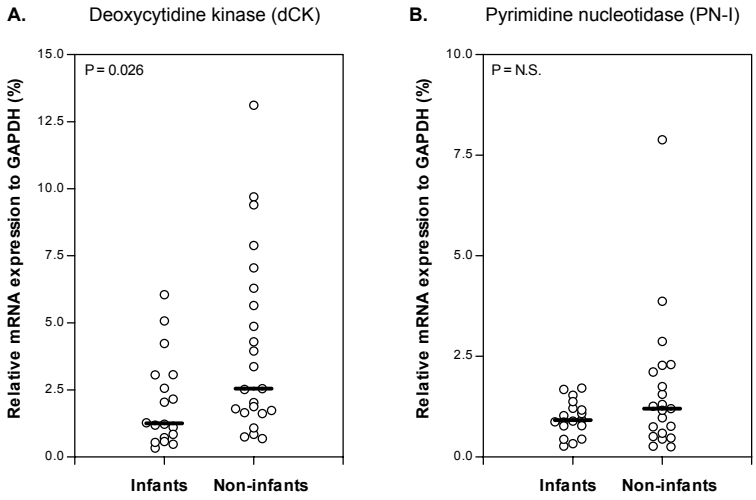

Figure 3. Relative *dCK* and *PN-I* mRNA expression (Taqman).
Relative mRNA expression of deoxycytidine kinase (dCK) and pyrimidine nucleotidase (PN-I) in infants and non-infants with ALL. The lines indicate the median values, circles (o) represent individual patients. N.S. = not significant.

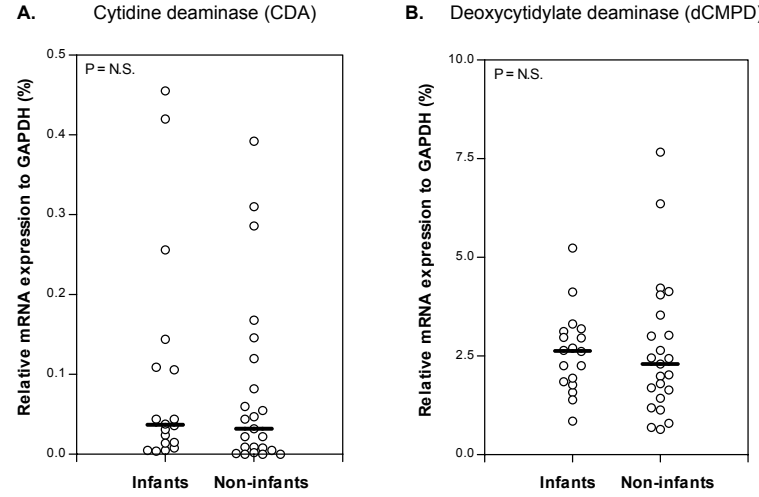

Figure 4. Relative CDA and dCMPD mRNA expression (Taqman).
Relative mRNA expression of cytidine deaminase (CDA) and deoxycytidylate deaminase (dCMPD) in infants and non-infants with ALL. The lines indicate the median values, circles (o) represent individual patients. N.S = not significant.

Infants expressed significantly less *dCK* mRNA (p=0.026) compared with older children with ALL. The difference in the median relative *dCK* mRNA expression in the infant and non-infant group is 2-fold. No significant difference in mRNA expression of *PN-I*, an enzyme opposing dCK activity, was found (Figure 3). Also, no significant differences were found in the mRNA expression of the deaminating (Ara-C inactivating) enzymes

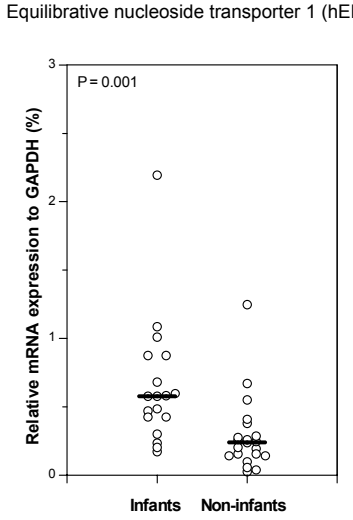

Figure 5. Relative *hENT1* mRNA expression (Taqman). Relative mRNA expression of the human equilibrative nucleoside transporter 1 (hENT1) in infants and non-infants with ALL. The lines indicate the median values, circles (o) represent individual patients.

CDA and *dCMPD* between infants and non-infants with ALL (Figure 4). However, mRNA of the nucleoside transporter *hENT1*, on which Ara-C is mainly dependent to cross the cell membrane, was significantly 2.5-fold higher expressed (p=0.001) in infants compared with non-infants with ALL (Figure 5).

Excluding the infant ALL samples from which the *MLL* status is unknown (n=2) and transferring the infant ALL samples with germ line *MLL* genes (n=2) to the non-infant (or rather *MLL* germ line) group (see Table 2), results in similar differences. *MLL* gene rearranged infant ALL patients express significantly 1.6-fold less *dCK* mRNA (p=0.043) and 2.5-fold more *hENT1* mRNA (p=0.001) compared to ALL patients with germ line *MLL* genes.

The oligonucleotide microarray screen comparing a group of *MLL* gene rearranged ALL patients (n=18), consisting of 15 infants and 3 older children, and a group of children older than 12 months of age with conventional ALL (n=23),[15] revealed similar results. No significant differences in mRNA expression between *MLL* gene rearranged ALL patients and ALL patients without *MLL* gene abnormalities were found for the deaminating enzymes CDA and dCMPD (data not shown). In contrast, the relative gene expression of *dCK* was significantly (p=0.001) lower (1.9-fold) and *hETN1* expression significantly (p=0.046) higher (2.7-fold) in *MLL* gene rearranged ALL patients (Figure 6). The expression of *PN-1* was not measured because no oligonucleotides representing this gene are present on the Affymetrix microarray chip used in this screen.

The *hENT1* mRNA expression inversely correlates with the LC_{50} values of Ara-C (r_s=-0.58, p=0.006) (Figure 7). In other words, Ara-C sensitivity correlates with increased *hENT1* mRNA expression. In contrast, we found that increased *dCK* mRNA expression tends to correlate, although weakly, with increased resistance to Ara-C (r_s=0.41, p=0.052).

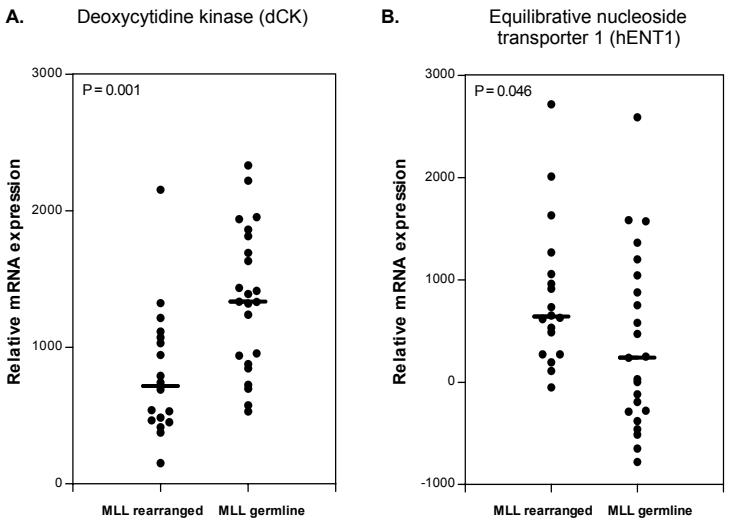

Figure 6. Relative *dCK* and *hENT1* mRNA expression (Microarray).
Deoxycytidine kinase (dCK) and equilibrative nucleoside transporter 1 (hENT1) mRNA expression in *MLL* gene rearranged (n=18) and *MLL* germ line (n=23) ALL patients measured on oligonucleotide microarrays. The lines indicate the median values, black dots represent individual patients.

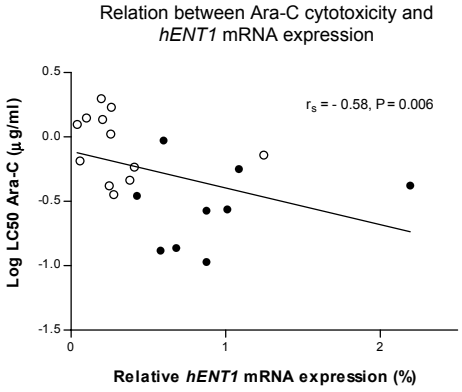

Figure 7. Relation between Ara-C cytotoxicity and *hENT1* mRNA expression (Taqman).
Correlation between the Ara-C cytotoxicity (log LC_{50} in ug/ml) and the relative mRNA expression of the human equilibrative nucleoside transporter 1 (hENT1). Open circles indicate individual infant ALL patients. Non-infants with ALL are indicated by black dots.

DISCUSSION

Infant ALL is characterized by a high incidence of *MLL* gene rearrangements and a poor treatment outcome compared to older children with ALL. The poor prognosis for infants with ALL is associated with rearrangements of the *MLL* gene,[4,21,22] and *in vitro*

resistance to prednisone and L-asparaginase.[7] However, infant ALL cells are highly sensitive to cytosine arabinoside (Ara-C).[7] This knowledge has recently been implemented in a new international collaborative treatment protocol, i.e. INTERFANT-99, for infants with ALL in order to give a more specific treatment to infant ALL patients.

The necessity of an exclusive treatment protocol for infants diagnosed with ALL has recently been stressed by a gene expression profiling study comparing (mostly) infants with *MLL* gene rearranged ALL, and older children with conventional ALL and acute myeloid leukemia (AML) without *MLL* gene rearrangements, using oligonucleotide microarrays.[15] This resulted in a gene expression profile for *MLL* gene rearranged ALL patients that is clearly distinguishable from the gene expression profiles found for conventional ALL and AML. This finding suggests that *MLL* gene rearranged leukemia needs to be classified as an unique leukemia for which urgently molecular targets need to be found to explore the possibilities of developing an appropriate treatment protocol for these patients.

In the present study we analyzed the mechanism of Ara-C sensitivity in infant *MLL* gene rearranged ALL. Because aberrant expression of key enzymes in Ara-C metabolism (Figure 1) might explain the remarkable sensitivity to Ara-C characteristic for these patients, we used quantitative real-time PCR analysis (Taqman chemistry) and an oligonucleotide microarray screen[15] to determine the mRNA levels of several Ara-C metabolizing enzymes in both infants and in older children with newly diagnosed ALL. Quantitative RT-PCR analysis revealed that infants with ALL express significantly 2-fold less *dCK* and 2.5-fold more *hENT1* mRNA compared with older children with ALL. No significant differences in *PN-I*, *CDA* and *dCMPD* mRNA expression were observed. The *hENT1* mRNA expression correlated with sensitivity to Ara-C, whereas *dCK* mRNA expression tended to correlate, although weakly, with Ara-C resistance. In approximately 80% of the infant ALL samples, the *MLL* gene is rearranged (Table 2). Comparison between *MLL* gene rearranged infants and *MLL* germ line patients revealed the same differences in *dCK* and *hENT1* expression as observed for the infant versus non-infant group. Similar results were obtained from the oligonucleotide microarray screen. *MLL* gene rearranged ALL patients expressed 1.9-fold less *dCK* and 2.7-fold more *hENT1* mRNA compared to children with ALL without *MLL* gene rearrangements. Because *MLL* gene rearrangements are strongly associated with infant ALL, it is difficult to determine whether the observed differences are related to *MLL* gene rearrangements or to infancy, or even to both. To answer this question, a large group of rather rare *MLL* germ line infant ALL samples is needed. However, since infant ALL is so strongly associated with *MLL* gene rearrangements, observations found in a group of infants with ALL shall therefore resemble a group of *MLL* gene rearranged ALL samples and vice versa.

Deoxycytidine kinase (dCK) generally phosphorylates deoxycytidine (dCyd) to form dCMP, but also phosphorylates a variety of deoxycytidine analogues, including Ara-C.[23] Reduced *dCK* mRNA expression and deficiency of functional dCK has often been associated with Ara-C resistance.[24,25,26,27] In addition, it has been demonstrated that relapsed ALL and AML patients show decreased *dCK* mRNA expression[28] and that initial childhood ALL patients expressing low levels of *dCK* mRNA are more likely to relapse than patients expressing higher levels of *dCK* mRNA.[29] After Ara-C is phosphorylated by dCK, Ara-CMP is further phosphorylated to Ara-CDP by (deoxy)cytidylate kinase (UMP-CMPK) and finally to its active, cytotoxic form Ara-CTP by nucleotide diphosphate kinases (NDPKs). Because dCK has the lowest cellular concentration of these three kinases, it is thought to be the rate-limiting enzyme in the activation of Ara-C[11]. However, we show that infants with ALL express significantly 2-fold less *dCK* mRNA than older children with ALL, whereas no significant differences in *PN-I* mRNA expression was found. Since PN-I opposes the action of dCK by dephosphorylating Ara-CMP,[12] the net phosphorylation of Ara-C into Ara-CMP may be considerably lower in infants with ALL. Yet, leukemic cells from these patients are significantly 3.3-fold more sensitive to Ara-C and 2.3-fold more Ara-CTP was formed upon exposure to Ara-C. Moreover, we observed that *dCK* mRNA expression tends to correlate, although weakly, with the obtained LC_{50} values for Ara-C. In other words, higher *dCK* mRNA expression tends to correlate with increased resistance to Ara-C. These data suggest that dCK is not a rate-limiting factor in the activation of Ara-C in infant ALL cells, which may support the findings of White *et al*[10] (see below). Another possibility could be that dCK is post-transcriptionally regulated and that despite of the lower mRNA expression, the amount of protein or the activity of the enzyme in infant ALL cells are comparable with, or even higher than in cells from older children with ALL.

Cytidine deaminase (CDA) and deoxycytidylate deaminase (dCMPD) both inhibit the formation of Ara-CTP by converting Ara-C to Ara-U and Ara-CMP to Ara-UMP respectively. Increased CDA and dCMPD activity in several cell lines transfected with human *CDA* or human *dCMPD* cDNA has been shown to confer resistance to Ara-C.[13,14,30,31] Furthermore, CDA activity proofed to be significantly higher in Ara-C refractory AML patients than in untreated patients.[32] Our results did not show significant differences in the mRNA expression of *CDA* and *dCMPD* between infants and older children with ALL, suggesting that Ara-C sensitivity in infant ALL can not be ascribed to decreased inactivation of Ara-C by these enzymes.

Nucleosides and their analogues are hydrophilic molecules, and therefore dependent on specialized transport proteins to permeate cell membranes. Ara-C membrane transport is mainly facilitated by a nitrobenzylmercaptopurine riboside (NBMPR) sensitive nucleoside transport system.[9,10,33] Since the human equilibrative nucleoside transporter 1 (hENT1) is the only known NBMPR sensitive nucleoside transporter capable of trans-

porting pyrimidine nucleosides over the cell membrane,[8] it is reasonable to assume that Ara-C mainly enters the cell via hENT1. In this study we show that infants with ALL express significantly 2.5-fold more *hENT1* mRNA compared to older children with ALL. In addition, we demonstrate a strong correlation between *hENT1* mRNA expression and Ara-C sensitivity. Others showed that inhibition of Ara-C membrane transport with NBMPR confers Ara-C resistance in cells from both ALL and AML patients.[9,10,33] Taken together, these data suggest that Ara-C sensitivity in infant ALL can, at least to some extent, be explained by a higher *hENT1* mRNA expression, possibly resulting in more Ara-C membrane transport sites and thus an elevated uptake of Ara-C into the cell. Furthermore these data suggest that *hENT1* mRNA expression may be a valuable predictor of Ara-C sensitivity in both infant and older ALL patients.

Our observation that Ara-C sensitivity may be a consequence of increased *hENT1* mRNA expression and the finding that dCK apparently is not rate-limiting in the formation of Ara-CTP in infants with *MLL* gene rearranged ALL, is in concordance with the findings of White and co-workers.[10] White *et al*, showed that Ara-C is phosphorylated by dCK almost as rapidly as it enters the cell at extracellular Ara-C concentrations below 1 μM, whereas at high Ara-C concentrations (>10 μM) unphosphorylated Ara-C accumulates inside cells. Thus at Ara-C concentrations <1 μM, the rate of Ara-C accumulation inside the cell is primarily determined by the transport rate or rather the number of transporter sites on the cell membrane (i.e. *hENT1* expression). At extracellular Ara-C concentrations exceeding 10 μM, dCK becomes the rate-limiting factor.[10] The median LC_{50} values for Ara-C for the infant and non-infant group in this study are 0.27 μg/ml (1 μM) and 0.89 μg/ml (3.6 μM) Ara-C respectively. Therefore, it is likely that the rate of Ara-C influx via hENT1 and not the level of dCK determines the amount of Ara-C phosphorylation by dCK and subsequently the sensitivity to Ara-C in these patient samples, even if dCK is post-transcriptionally regulated and the actual protein levels or enzyme activity in the infant samples are comparable or even higher than in cells from older children. Since infants with ALL who enter the INTERFANT-99 treatment protocol receive both low-dose and high-dose Ara-C, corresponding to plasma levels <1 μM and >10 μM respectively[34,35], these patients may benefit from their increased *hENT1* expression at least during treatment with low to normal doses of Ara-C.

Based on these considerations we conclude that the observed increase in *hENT1* mRNA expression contributes to the remarkable sensitivity to Ara-C in infants diagnosed with ALL.

ACKNOWLEDGEMENTS

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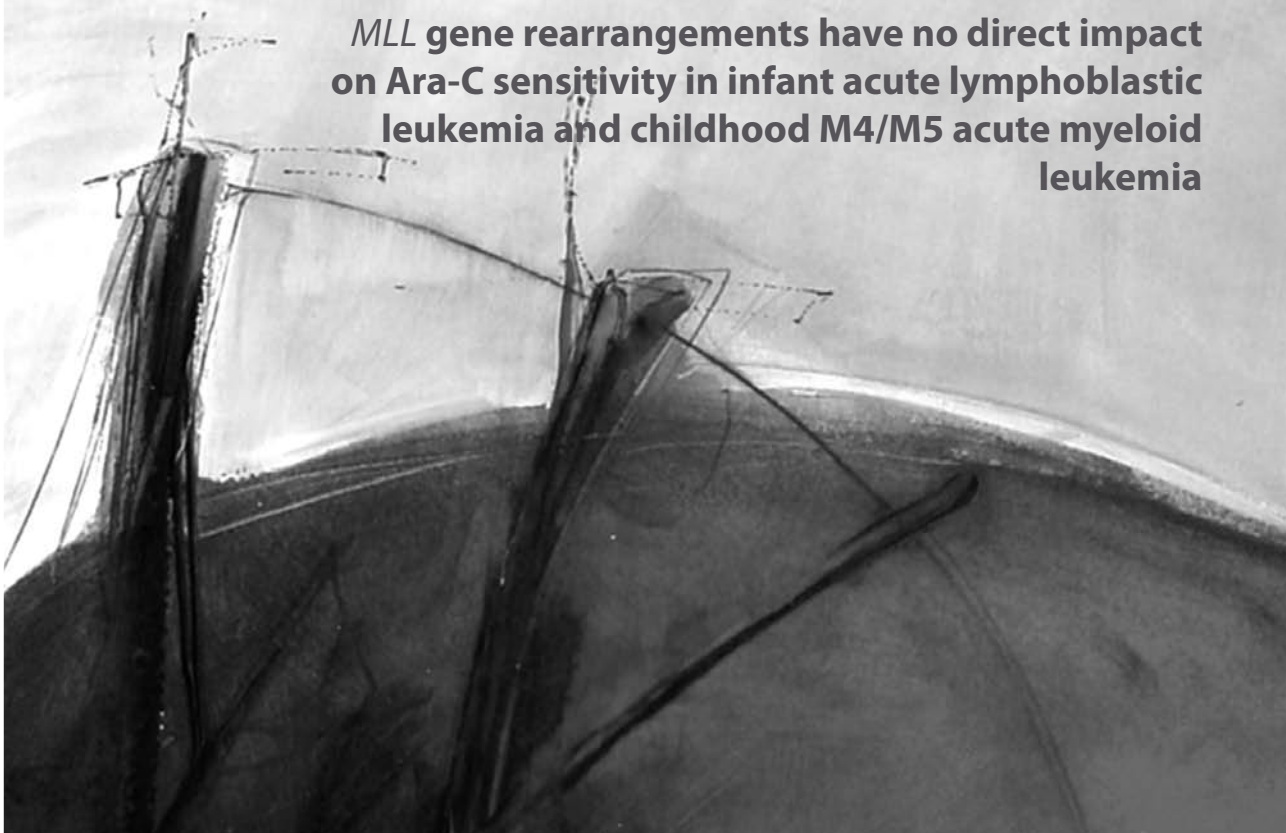
Chapter 4

MLL gene rearrangements have no direct impact on Ara-C sensitivity in infant acute lymphoblastic leukemia and childhood M4/M5 acute myeloid leukemia



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Leukemia (in press)

TO THE EDITOR

The anti-metabolite cytosine arabinoside (Ara-C) is a deoxycytidine analogue that is used as a therapeutic agent in many leukemia treatment regimens. In combination with anthracyclines, Ara-C is the most effective agent in the treatment of acute myeloid leukemia (AML). In the treatment of acute lymphoblastic leukemia (ALL) the use of Ara-C is limited. However, leukemic cells from infants (<1 year of age) with ALL, which are resistant to several chemotherapeutic drugs, are *in vitro* more sensitive to Ara-C as compared to older children with ALL.[1,2] This observation suggested that infant ALL might resemble a subclass of childhood ALL which may benefit from intensified treatment with Ara-C to improve the dismal prognosis for these patients who to date experience an event free survival (EFS) of ~35%. Therefore, in 1999 a novel collaborative treatment protocol (INTERFANT-99) was designed with intensified use of Ara-C, in order to provide a more specific treatment to infant ALL patients.

Ara-C mainly depends on the human equilibrative nucleoside transporter 1 (ENT1) to permeate the cell membrane. Inside cells, the pro-drug Ara-C is phosphorylated into

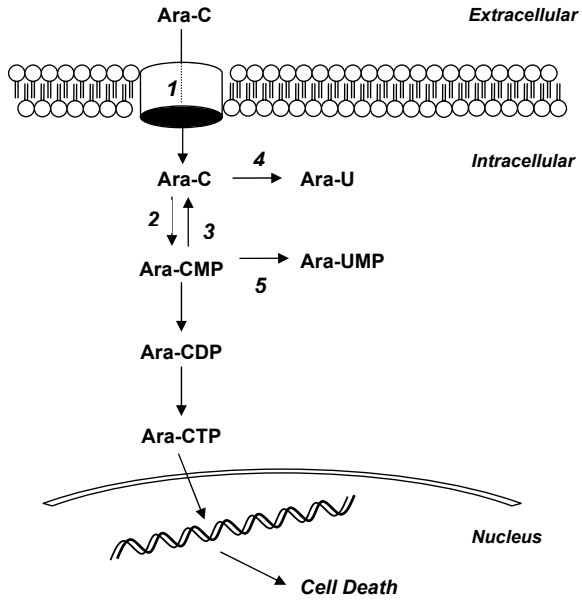

Figure 1. Ara-C metabolism.

Ara-C enters the cell mainly via *(1)* equilibrative nucleoside transporter 1 (hENT1) and is subsequently phosphorylated to form Ara-CMP by *(2)* deoxycytidine kinase (dCK), followed by two more phosphorylation steps catalyzed by (deoxy)cytidylate kinase (UMP-CMPK) and nucleoside diphosphate kinases (NDKs), respectively, to form Ara-CTP. Incorporation of Ara-CTP into the DNA during DNA synthesis leads apoptosis. Ara-CTP formation can, however, be obstructed. *(3)* Pyrimidine nucleotidase I (PN-I) inhibits Ara-CTP formation by opposing the action of dCK. *(4)* Cytidine deaminase (CDA) and *(5)* deoxycytidylate deaminase (dCMPD) both are inactivating enzymes, which convert Ara-C to Ara-U and Ara-CMP to Ara-UMP, respectively, thereby decreasing the amount of functional Ara-CTP that can be formed.

Table 1. Patient characteristics.

	Infant ALL	Childhood AML
Number (n)	34	27
Sex (♂:♀) (%)	41 : 59	61 : 39
Age, yrs. (median; P25-P75)[§]	0.47 (0.24 – 0.76)	10.4 (2.87 – 14.57)
Immunophenotype (%) :		
AML : M4	-	61
M5	-	39
ALL : Pro-B	68	-
Common	17	-
Pre-B	6	-
Pro-T	3	-
AUL	3	-
Unknown	3	-
MLL gene status (%) :		
t(4;11)	47	-
t(11;19)	29	-
t(9:11)	-	29
11q23*	-	21
Germ line	24	50

[§]P25-P75 indicate the 25th and 75th percentiles

*11q23 indicates translocations involving *MLL* other than t(4;11), t(11;19), or t(9;11).

its active, cytotoxic form Ara-CTP, as which it competes with dCTP for incorporation into the DNA. When incorporated, Ara-C blocks DNA synthesis and induces apoptosis (Figure 1). Ara-C is phosphorylated by deoxycytidine kinase (dCK) to form Ara-CMP. Subsequently, Ara-CTP is formed by two additional phosphorylations catalyzed by (deoxy)cytidylate kinase (UMP-CMPK) and nucleoside diphosphate kinases (NDKs) respectively (Figure 1). Several enzymes, however, oppose the formation of functional Ara-CTP, and thus the cytotoxic effects of this drug. Pyrimidine nucleotidase I (PN-I) for example preferentially dephosphorylates Ara-CMP to Ara-C.[3] Cytidine deaminase (CDA) and deoxycytidylate deaminase (dCMPD) convert Ara-C to Ara-U and Ara-CMP to Ara-UMP respectively, by deamination of the cytosine base of Ara-C. Recently we demonstrated that Ara-C sensitivity in infant ALL is related to increased expression of *ENT1* (responsible for Ara-C transport across the cell membrane[4]), and not to the expression of other genes involved in Ara-C metabolism (mentioned above).

Infant ALL is characterized by rearrangements of the *Mixed Lineage Leukemia* (*MLL, ALL-1, or HRX)* gene on chromosome band 11q23, in approximately 80% of the cases. The most common translocations involving *MLL* in infant ALL are t(4;11)(q21;q23) and t(11;19)(q23;p13.3) resulting in a fusion of *MLL* with *AF-4* on chromosome 4 and *ENL* on chromosome 19, respectively. In childhood acute myeloid leukemia (AML),

MLL gene rearrangements are mainly restricted to the FAB M4 and M5 subtypes, and occur in the majority of the infant cases. Interestingly, childhood M4/M5 AML harboring t(9;11)(q22;q23) in which *MLL* is fused to *AF-9* on chromosome 9, has also been reported to be highly sensitive to Ara-C when compared to other AML subtypes without *MLL* rearrangements.[5] The fact that Ara-C sensitivity is found in both *MLL* rearranged infant ALL as well as *MLL* rearranged M4/M5 AML, raises an important question: is Ara-C sensitivity (or increased expression of *ENT1*) directly associated with rearrangements of the *MLL* gene? To answer this question we set out to determine the relationship between Ara-C sensitivity, expression of Ara-C metabolizing enzymes, and the presence of *MLL* gene rearrangements in both infant ALL and pediatric AML M4/M5. Patient characteristics are listed in Table 1.

The MTT-assay was used to determine *in vitro* Ara-C cytotoxicity. Within infant ALL, no significant difference in Ara-C sensitivity was found between *MLL* rearranged (MLL^+) and *MLL* germ line (MLL^-) infant ALL cases (Figure 2a). Furthermore, no differences in Ara-C sensitivity were observed between infant ALL patients harboring either translocation t(4;11) or t(11;19) as compared to *MLL* germ line cases (Figure 2b). Also, no statistically significant difference in Ara-C cytotoxicity was observed between MLL^+ and MLL^- AML M4/M5 cases (Figure 3a), nor between AML M4/M5 patients carrying t(9;11) or other translocations involving *MLL* (11q23) compared to AML M4/M5 patients carrying germ line *MLL* genes (Figure 3b).

The relative mRNA expression levels of *ENT1*, *dCK*, *PN-I*, *CDA* and *dCMPD* was measured using quantitative real-time PCR (TaqMan) as described before.[4] The expression of genes important for membrane transport, and for the metabolism of Ara-C into its active, cytotoxic form Ara-CTP, did not significantly differ between MLL^- and MLL^+ infant ALL cases (Table 2), nor between MLL^- and MLL^+ M4/M5 AML cases (Table 3). The only exception was *PN-I*, which was significantly higher expressed in infant ALL patients carrying germ line *MLL* genes as compared to MLL^+ infant ALL samples (Table 2). This would be in line with the hypothesis that *MLL* rearrangements induce Ara-C sensitivity, as this would imply that increased dephosphorylation of Ara-CMP leads to increased resistance to Ara-C in leukemic cells from infant ALL patients carrying germ line *MLL*. However, increased *PN-I* expression does not seem to affect Ara-C sensitivity in these patients, as no significant difference in Ara-C cytotoxicity could be observed between both patient groups (Figure 2).

Our finding that Ara-C sensitivity is not directly associated with the presence of *MLL* rearrangements is in agreement with our recent study.[2] No apparent differences in Ara-C sensitivity were found between MLL^- and MLL^+ ALL cases <1 year of age, nor between MLL^- and MLL^+ ALL above this age.[2] Moreover, the same study further demonstrated no statistical differences in Ara-C cytotoxicity between t(4;11) positive ALL samples and samples carrying other translocations involving *MLL*.[2] Furthermore, it appeared that among ALL patients ≥1 year of age, pro-B ($CD10^-$, $CD19^+$) cases are

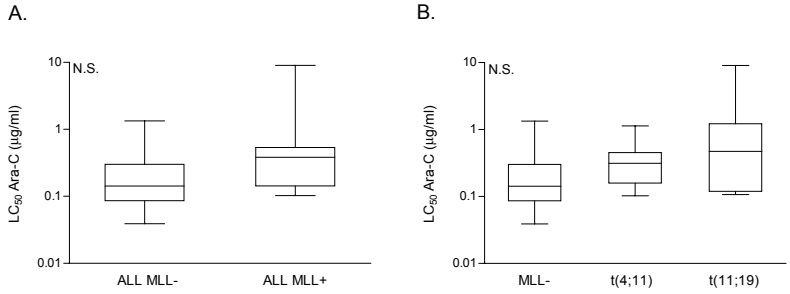

Figure 2. Ara-C cytotoxicity in infant ALL.

In vitro Ara-C cytotoxicity (LC_{50} Ara-C in µg/ml) in **a.** leukemic cells from *MLL* gene rearranged (n=26) and *MLL* germ line (n=8) infant ALL patients. Possible differences between both patient groups were statistically analyzed using the Mann-Whitney U test. **b.** *In vitro* Ara-C cytotoxicity in leukemic cells from infant ALL patients carrying either translocation t(4;11) (n=16), t(11;19) (n=10) or germ line *MLL* (n=8). Possible differences between these patient groups were statistically analyzed using the Kruskal-Wallis test. The box plots indicate the group medians, 25th/75th percentiles and the ranges of Ara-C cytotoxicity.

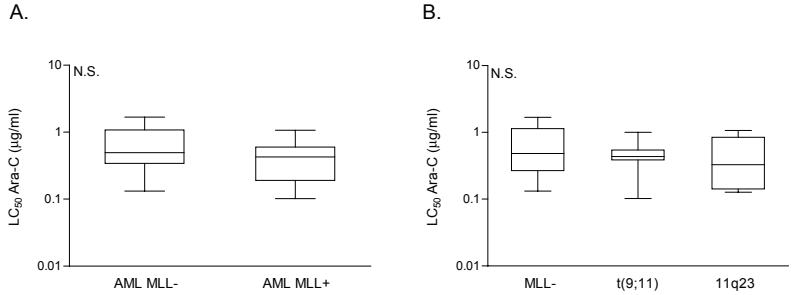

Figure 3. Ara-C cytotoxicity in childhood M4/M5 AML.

In vitro Ara-C cytotoxicity (LC_{50} Ara-C in µg/ml) in **a.** leukemic cells from *MLL* gene rearranged (n=14) and *MLL* germ line (n=13) childhood M4/M5 AML patients. Possible differences between both patient groups were statistically analyzed using the Mann-Whitney U test. **b.** *In vitro* Ara-C cytotoxicity in cells from childhood M4/M5 AML patients carrying either translocation t(9;11) (n=8), unknown translocation involving *MLL* (11q23) (n=6) or germ line *MLL* (n=13). Possible differences between these patient groups were statistically analyzed using the Kruskal-Wallis test. The box plots indicate the group medians, 25th/75th percentiles and the ranges of Ara-C cytotoxicity.

more sensitive to Ara-C as compared to common ($CD10^+$, $CD19^+$) and pre-B ($CD19^+$, $CyIg\mu^+$) ALL cases. However, in infant ALL patients (<1 year of age) the pro-B phenotype was not associated with sensitivity to Ara-C, but the number of infants with pre-B or common phenotypes was small.[2] Thus, Ara-C sensitivity in ALL seems to be associated with age (i.e. <1 year) and immunophenotype, but not with the presence of *MLL* rearrangements. Possibly, sensitivity to Ara-C as observed in childhood AML M4/M5 cells is associated with the M4/M5 FAB type rather than with young age.

In conclusion, the present study does not provide data to support that *MLL* rearrangements are directly involved in the increased sensitivity to Ara-C as detected in infant ALL and M4/M5 AML.

Table 2. Comparison of expression levels of Ara-C metabolizing enzymes between infant ALL patients with and without *MLL* gene rearrangements.

Gene	Relative mRNA expression (%)	*MLL* rearranged (n=22)	*MLL* germ line (n=6)	Expression ratio†
ENT1	median P25 – P75§	0.174 0.082 – 0.32	0.103 0.074 – 0.12	1.69
dCK	median P25 – P75§	0.483 0.284 – 0.882	0.869 0.273 –3.376	0.56
PN-I	median P25 – P75§	0.288 0.226 – 0.34	0.9736 0.477 – 1.167	0.3*
CDA	median P25 – P75§	0.006 0.0008 – 0.015	0.003 0.0003 – 0.045	2
dCMPD	median P25 – P75§	0.822 0.481 – 1.168	0.756 0.433 – 1.369	1.09

§P25 and P75 indicate the 25th and 75th percentiles respectively

†Median expression in *MLL* rearranged patie-nts divided by the median expression in *MLL* germ line patients. Values >1.0 indicate higher median expression in the *MLL* rearranged patient group.

P=0.001 as determined by the Mann-Whitney *U* test

Table 3. Comparison of expression levels of Ara-C metabolizing enzymes between childhood AML M4/M5 patients with and without *MLL* gene rearrangements.

Gene	Relative mRNA expression (%)	*MLL* rearranged (n=13)	*MLL* germ line (n=12)	Expression ratio†
ENT1	Median P25 – P75§	0.975 0.701 – 1.752	0.714 0.567 – 1.223	1.37
dCK	Median P25 – P75§	0.489 0.188 – 0.835	0.363 0.238 – 0.517	1.35
PN-I	Median P25 – P75§	0.297 0.106 – 0.767	0.343 0.15 – 0.55	0.87
CDA	Median P25 – P75§	0.149 0.055 – 0.44	0.22 0.05 – 0.505	0.68
dCMPD	Median P25 – P75§	0.989 0.637 – 1.931	0.921 0.775 – 1.517	1.07

§P25 and P75 indicate the 25th and 75th percentiles respectively

†Median expression in *MLL* rearranged patients divided by the median expression in *MLL* germ line patients. Values >1.0 indicate higher median expression in the *MLL* rearranged patient group.

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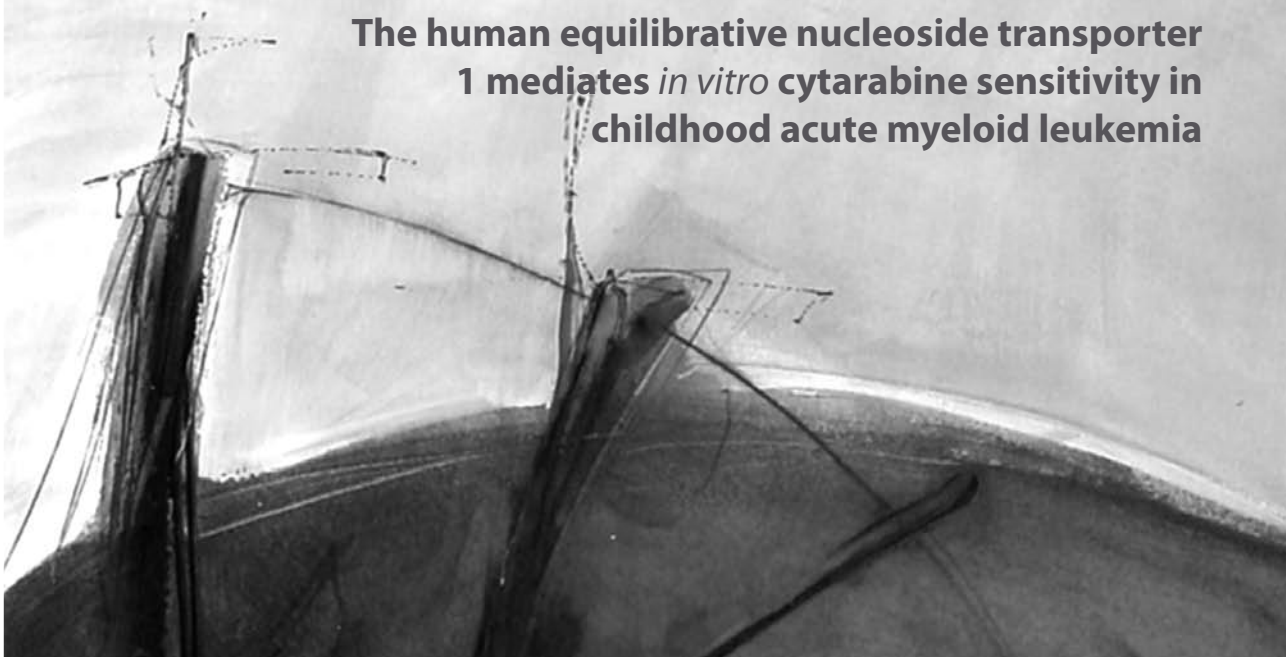
Chapter 5

The human equilibrative nucleoside transporter 1 mediates *in vitro* cytarabine sensitivity in childhood acute myeloid leukemia



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ABSTRACT

Cytarabine (ara-C) is the most effective agent for the treatment of acute myeloid leukemia (AML). Aberrant expression of enzymes involved in the transport/metabolism of ara-C may potentially explain resistance to this drug. We determined mRNA expression of these factors using quantitative-real-time-PCR in leukemic blasts from children diagnosed with *de novo* AML. Expression of the inactivating enzyme pyrimidine nucleotidase-I (PN-I) was 1.8-fold lower in FAB-M5 as compared to FAB-M1/2 (p=0.007). *In vitro* sensitivity to deoxynucleoside analogs was determined using the MTT-assay. Human equilibrative nucleoside transporter 1 (*hENT1*) mRNA expression and ara-C sensitivity were significantly correlated (r_p=-0.46, p=0.001), with 3-fold lower *hENT1* mRNA levels in resistant patients (p=0.003). *hENT1* mRNA expression also seemed to correlate inversely with the LC_{50} values of cladribine (r_p=-0.30; p=0.04), decitabine (r_p=-0.29; p=0.04) and gemcitabine (r_p=-0.33; p=0.02). Deoxycytidine kinase (*dCK*) and cytidine deaminase (*CDA*) mRNA expression seemed to correlate with *in vitro* sensitivity to gemcitabine (r_p=-0.31; p=0.03) and decitabine (r_p=0.33; p=0.03), respectively. The *dCK*/*PN-I* expression ratio correlated inversely with LC_{50} values for gemcitabine (r_p=-0.45, p=0.001) and the *dCK*/*CDA* expression ratio seemed to correlate with LC_{50} values for decitabine (r_p=-0.29; 0.04). In conclusion, decreased expression of hENT1, which transports ara-C across the cell membrane, appears to be a major factor in ara-C resistance in childhood AML.

INTRODUCTION

Although the treatment of acute leukemia has improved significantly over the past few decades, the prognosis of acute myeloid leukemia (AML) remains relatively poor. For newly diagnosed patients the compete remission (CR) rate reaches 85-90% with standard induction chemotherapy.[1,2] However, about 30-50% of the patients that achieve CR relapse from minimal residual disease cells that apparently survived chemotherapy,[2] giving rise to a more resistant leukemia. Resistance to chemotherapy therefore remains a major obstacle in the treatment of AML.

In combination with anthracyclines, 1-ß-D-arabinofuranosylcytosine (cytosine arabinoside; cytarabine; ara-C), is the most effective agent for the treatment of AML. Ara-C is a deoxynucleoside analog that has to be converted into its active triphosphate derivative (ara-CTP) to exert its cytotoxic effect.[3] Ara-CTP is then incorporated into the DNA causing chain termination, resulting in a block in DNA synthesis, and facilitates programmed cell death (Figure 1). Ara-C is a hydrophilic molecule and as such requires facilitated diffusion via nucleoside-specific membrane transport carriers to enter cells.[4,5] The human equilibrative nucleoside transporter (hENT1) is responsible for ~80% of ara-C influx in human leukemic blast cells.[5,6] Inside the cell, conversion of ara-C into ara-CMP by deoxycytidine kinase (dCK) is believed to be the rate-limiting step in the metabolism of ara-C.[7,8] Subsequently, ara-CMP is phosphorylated into ara-CDP by nucleoside monophosphate kinases, which in turn is finally phosphorylated into ara-CTP by diphosphate kinases.[9] Inactivation of ara-C results from deamination by cytidine deaminase (CDA).[10] In addition, ara-CMP can be dephosphorylated by pyrimidine nucleotidase I (PN-I)[11] as well as deaminated by deoxycytidylate deaminase (dCMPD).[12] Inactivation by these enzymes decreases the amount of ara-CTP that can be formed, and thereby limits ara-C mediated cytotoxicity. Deoxycytidine triphosphate (dCTP) is thought to inhibit the phosphorylation of ara-C (by feedback inhibition of dCK) and the incorporation of ara-CTP into DNA (by competition for DNA polymerase). Increased intracellular dCTP pools therefore antagonize the formation of ara-CTP.[13,14] dCTP can be synthesized directly via the *de novo* pathway by ribonucleotide reductase (RR).[15] RR catalyzes the conversion of ribonucleotides into deoxyribonucleotides.[15] Mammalian RR is made up out of two subunits.[16] The M1 subunit harbors the binding site for nucleotides and the second subunit, M2, contains a metal binding site that requires both a non-heme iron and a tyrosine free radical for its activity.[15] CTP synthetase (CTPs) is responsible for the conversion of uridine triphosphate (UTP) into CTP and has high activity in several malignancies, including acute lymphoblastic leukemia.[17]

In addition to ara-C, a variety of other deoxynucleoside derivatives are active in both hematological and solid malignancies. The purine analogs 2-chlorodeoxyadenosine (cladribine; 2-CdA) and fludarabine (F-ara-A) are active against indolent lymphoid

malignancies and are currently also used for the treatment of hairy-cell leukemias, and both chronic and acute leukemias, respectively.[18] The pyrimidine analog gemcitabine (dFdC) has activity in various solid malignancies and some hematological disorders.[19] The cytidine analog 5-aza-2'–deoxycytidine (decitabine; DAC) is a potent hypomethylating agent and has shown to be active in the treatment of hematological malignancies such as AML, chronic myeloid leukaemia (CML) and myelodysplastic syndrome.[20] These compounds are activated intracellularly via the same metabolic pathway as ara-C. Impaired transport, decreased activation of deoxynucleoside analogs to their cytotoxic tri-phosphate form, or increased dCTP levels may result in resistance to this clinically important group of compounds. The objective of our study was to identify possible mechanisms of resistance to deoxnucleoside analogs, particularly ara-C, in the leukemic blasts of pediatric AML patients. We therefore determined the mRNA level of the following targets: *hENT1, dCK, PN-I, CDA, dCMPD, CTPs,* and *RR* (subunit 1 and 2) in leukemic blasts from children with newly diagnosed AML. In addition, we studied the mRNA expression levels of the target enzymes in different AML FAB-type subgroups. Finally, the expression levels of the above mentioned enzymes were correlated to *in vitro* sensitivity to deoxynucleoside analogs (ara-C, 2-CdA, DAC, F-ara-A and dFdC).

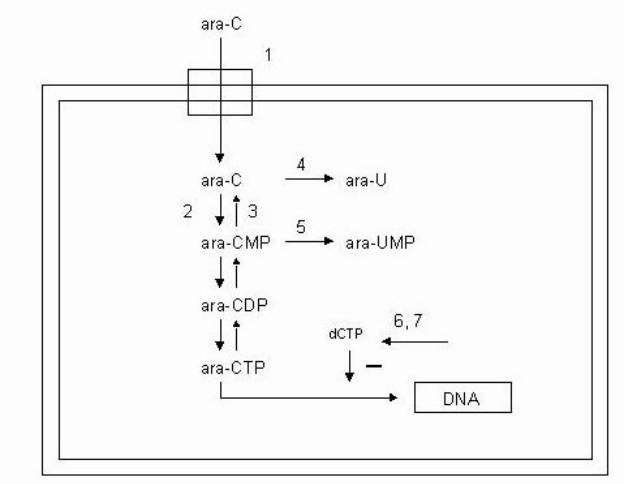

Figure 1. Metabolism of ara-C.

Ara-C enters the cell mainly via the equilibrative nucleoside transporter 1 (hENT1; 1). Inside the cell, ara-C is phosphorylated into ara-CMP by deoxycytidine kinase (dCK; 2). Ara-CMP is subsequently phosphorylated to ara-CTP, the active metabolite. Incorporation of ara-CTP into the DNA blocks DNA synthesis and leads to programmed cell death (apoptosis). Ara-CTP formation can, however, be obstructed. Pyrimidine nucleotidase I (PN-I; 3) opposes the action of dCK by dephosphorylating ara-CMP to form ara-C. Cytidine deaminase (CDA; 4) and deoxycytidylate deaminase (dCMPD; 5) convert ara-C to ara-U, and ara-CMP to ara-UMP, respectively, decreasing the amount of functional ara-CTP that can be formed. Furthermore, increased intracellular dCTP pools are believed to antagonize the formation of ara-CTP. Both ribonucleotide reductase (6) and CTP synthetase (CTPs; 7) are involved in *de novo* dCTP synthesis. CTPs is capable of converting uridine triphosphate (UTP) into CTP. CTP can either become incorporated into RNA, or become deposphorylated to form CDP. In turn, ribonucleotide reductase converts CDP into dCDP, which competes with Ara-CDP for their final phosphorylation into triphosphates (dCTP and ara-CTP). As such, increased expression of ribonucleotide reductase and CTPs inhibit ara-CTP formation either by competition or by feedback inhibition as a result of increasing dCTP pools.

MATERIALS AND METHODS

Patient samples

Bone marrow and/or peripheral blood samples were collected from untreated children diagnosed with *de novo* AML. The following groups participated in this study and provided patient samples: (1) The Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands; (2) MRC Childhood Leukaemia Working Party, UK and (3) The AML BFM-study Group, Münster, Germany. Central review of the diagnosis, data collection as well as review of FAB-classification was performed by reference laboratories and data centers of these groups. The FAB-classification was performed according to the criteria by Bennett et al, including the modifications to diagnose FAB M0 and FAB M7.[21] Samples were collected at the VU university medical center between October 1990 and September 2002.

Treatment protocols

Patients were treated on intensive ara-C/anthracyclines based protocols in the Netherlands, Germany and the UK (protocols DCOG AML 87 and 97, BFM 93 and 98 and MRC AML 12). The treatment protocols have been reported in detail elsewhere.[1,2,22,23]

In the AML BFM 93 study, the patients were stratified according to risk group. At diagnosis, patients were randomized between daunorubicin (plus ara-C and etoposide, ADE) and idarubicin (plus ara-C and etoposide, AIE) induction therapy. For high-risk (HR) patients, one of the intensification blocks was changed to high-dose ara-C with mitoxantrone (HAM). Sibling stem cell transplantations (SCT) was advised for HR patients in first CR. Standard risk (SR) patients did not receive HAM. Protocol AML BFM 98 consisted of induction with the idarubicin block, followed by HAM. In the consolidation phase patients were randomized for either receiving the 6-week consolidation block followed by one intensification block vs. three intensive courses of chemotherapy.

The DCOG AML 87 protocol was based on the concurrent AML-BFM protocol. In brief, DCOG AML 87 started with an 8-day induction course followed by a 6-week consolidation block. Then two intensification courses were given. Intrathecal chemotherapy was given as central nervous system prophylaxis. In contrast to the AML BFM 87 study, no maintenance therapy was given. Sibling donor allogeneic STC was advised for HR patients in first CR.

Patients enrolled in the DCOG AML 97, which was identical to the MRC AML12 protocol, were stratified according to cytogenetics. Good risk patients (defined as patients with t(8;21), inv(16) or t(15;17)) were not eligible for SCT. Patients were randomized to induction treatment with either ADE (ara-C, daunorubicin and etoposide) or MAE (mitoxantrone, ara-C and etoposide), followed by a 4 or 5 (randomized) treat-

ment courses. The fifth course was high-dose ara-C and L-asparaginase. If a matched sibling donor was available, then SCT was recommended as the fourth or fifth course (randomized).

Leukemic cells

Mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/ml; Nycomed Pharma, Oslo, Norway), and centrifuged at 480 g for 15 minutes at room temperature. Cells were washed and resuspended in culture medium consisting of RPMI 1640 medium (Dutch modification without L-glutamine; Gibco BRL, Life Technologies, Breda, The Netherlands), 20% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Gibco BRL, Life Technologies), 5 µg/ml transferrin, 5 ng/ml sodium selenite (ITS media supplement; Sigma, St Louis, MO), 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.125 µg/ml fungizone (Gibco BRL, Life technologies), and 0.2 mg/ml gentamycin (Gibco BRL, Life technologies). Contaminating normal cells were removed by immunomagnetic beads (in case of lymphocytes) or by freezing in liquid nitrogen and thawing (in case of granulocytes).[24] All samples contained at least 80% leukemic cells, as determined morphologically on May-Grunwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. A minimum of $5x10^6$ cells were lysed in RNAzol or Trizol reagent (Gibco BRL, Life technologies) and stored at –80°C until RNA extraction. The majority of samples were received and processed within 24 hours (n=42), 8 samples, however, were received and processed within 48 hours.

RNA extraction and cDNA synthesis

Total cellular RNA was isolated from $5x10^6$ cells using RNAzol or Trizol reagent according to the manufacturer's protocol. After precipitation with ethanol, RNA pellets were dissolved in water. The RNA was quantified spectrophotometrically. cDNA synthesis was performed as described by Stam *et al*.[25] Briefly, following a denaturation step of 5 minutes at 70°C, 1 µg of RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers (2.5 µM) and oligo dT primers (20 nM). The RT reaction was performed in a total volume of 25 µl containing 0.2 mM of each dNTP (Amersham Pharmacia, Biotech, Piscataway, NJ) 200 U Moloney murine leukemia virus reverse transcriptase (M-ML RT; Promega, Madison, WI), and 25 U RNAsin (Promega) at 37°C for 30 minutes, 42°C for 15 minutes and 94°C for 5 minutes. The obtained cDNA was diluted to a final concentration of 8 ng/µl. Samples were stored at –80°C.

Quantitative real-time PCR (Taqman)

The mRNA expression levels of *dCK, PN-I, CDA, dCMPD, hENT1, RR1* and *RR2, CTPs,* and the endogenous housekeeping gene encoding glyceraldehyde-3-phosphate

dehydrogenase (*GAPDH*) as a reference, were quantified using real-time PCR analysis (Taqman) on an ABI Prism 7700 sequence detection system (PE Applied Biosystems). Amplification of specific PCR products was detected using dual-fluorescent non-extendable probes labeled with 6-carboxyfluorescein (FAM) at the 5' end, and with 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. All primers and probe combinations were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO) and purchased from Eurogentec (Seraing, Belgium). Primers and probes used to detect *hENT1, dCK, PN-I, CDA* and *dCMPD* have been reported before.[25] For *CTPs*, *RR1* and *RR2* primers and probes are listed in Table 1.

Table 1. Primers and probe combinations used for quantitative real-time PCR (Taqman).

Gene	Primer/probe	Sequence
RR1	forward	5'-GTG TGG GAA ATC TCT CAG A-3'
	reverse	5'-CCA TGG CTG CTG TGT T-3'
	probe	5'-(FAM)-CAA ACT CAC TAG TAT GCA CTT CTA CGG-(TAMRA)-3'
RR2		
	forward	5'-AGG GGC TCA GCT TGG-3'
	reverse	5'-GGG GCA GCT GCT TTA G-3'
	probe	5'-(FAM)-CGT CCT GGC CAG CAA GAC-(TAMRA)-3'
CTPs	forward	5'-ATC CCG TGG TCG TAG AC-3'
	reverse	5'-TGG CCA ACA AAC TTC AA-3'
	probe	5'-(FAM)-AAC ACA ACC CAG GGC AGA TG-(TAMRA)-3'

RR1: Ribonucleotide reductase subunit 1, RR2: Ribonucleotide reductase subunit 2,
CTPs: CTP synthetase.

As described before,[25] real-time PCR was performed in a total reaction volume of 50 µl containing TaqMan buffer A (Applied Biosystems), 4 mM $MgCl_2$, 200 µM of each dNTP (Amersham Pharmacia Biotech), 300 nM forward and reverse primer, 50 nM dual-labeled fluorogenic internal probe, 1.25 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems) and 40 ng of cDNA as a template. Samples were heated for 10 minutes at 95°C to activate the Ampli*Taq* Gold DNA polymerase and amplified during 40 cycles of 15 secondes at 95°C and 60 seconds at 60°C. The relative mRNA expression levels of the target genes in each patient were calculated using the comparative cycle time (C_t) method.[26] Briefly, this PCR C_t value is the cycle number at which emitted fluorescence exceeds 10x the standard deviation (SD) of baseline emissions as measured from cycles 3-15. The C_t of the target gene is normalized to the GAPDH PCR C_t value by subtracting the GAPDH C_t value from the target C_t value. The mRNA expression level for each target PCR relative to *GAPDH* was calculated using the following equation:

$$\text{relative mRNA expression} = 2^{-(\text{Ct target} - \text{Ct GAPDH})} \times 100\%.$$

In vitro cytotoxicity assay

In vitro cytotoxicity of the deoxynucleoside analogs ara-C (Cytosar; Pharmacia &Upjohn, Woerden, The Netherlands), 2-CdA (Leustatin, Ortho Biotech, USA), DAC (Decitabine, kindly provided by PCH Pharmachemie bv, Haarlem, The Netherlands), F-ara-A (Fludara, Schering AG, The Netherlands), Gemcitabine (Gemzar, Eli Lilly, Houten, The Netherlands) was determined using the MTT-assay as described previously.[27] Briefly, cells were cultured in round-bottomed 96-well microtitre plates in the presence of 6 concentrations of different drugs, in the following ranges: ara-C (0.04-41.0 μM); 2-CdA (0.001-140.0 μM); DAC (11.0 μM–11.0 mM); F-ara-A (0.04-44.0 μM) and dFdC (0.04-13.0 mM). Cells without drugs were included as controls and wells containing culture medium only were used as blanks. The plates were cultured for 4 days at 37°C in humidified air containing 5% CO_2, after which 10 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazoliumbromide (MTT; 5 mg/ml, Sigma Aldrich, Zwijndrecht, The Netherlands) was added and the plates were incubated for an additional 6 hours. Only viable cells are able to reduce MTT tetrazolium salt to purple/blue formazan crystals. The formazan crystals were dissolved using acidified isopropanol (0.04 N HCl-isopropyl alcohol) and the optical density (OD), which is linearly related to the number of viable cells,[28] was measured spectrophotometrically at 562 and 720 nm. After subtraction of the blank values, the leukemic cell survival (LCS) was calculated by the following equation:

$$LCS = (OD_{day4} \text{ treated well/mean } OD_{day4} \text{ control wells}) \times 100\%.$$

Drug sensitivity was expressed as the LC_{50} value, the drug concentration lethal to 50% of the leukemic cells. Reliable results were obtained when a minimum of 70% leukemic cells was present at day 4 and when the control OD was more than or equal to 0.05.[29] Sample source (bone marrow or peripheral blood) and cryo-preservation do not influence the results obtained by cellular drug resistance testing and were therefore analyzed together.[30]

Statistics

The distribution of measured values was characterized by median values and quartiles (25^{th}-75^{th} percentiles). Due to the strongly skewed character of the distributions, analyses were performed on log-transformed measurements. For significance, a two-tailed level of α=0.01 was used. P-values between 0.01 and 0.05 were considered to indicate a trend for significance. Pearson correlations were used to describe relations between variables. AML patient samples were divided into thee sub-groups according to their sensitivity to ara-C: sensitive (LC_{50} <0.98 μM), intermediate (0.98< LC_{50} <5.18 μM) and resistant (LC_{50} >5.18 μM) (the cut-off values are based on Zwaan *et al* (2000)[31], and a

one-way Anova test was performed on *hENT1* expression for these three sensitivity groups. Stepwise modeling on the log-transformed LC_{50} values was used to unravel the relative importance of the possible indicators.

RESULTS

Patient characteristics

Fifty-five AML patient samples with LC_{50} values for ara-C, cladribine, decitabine, fludarabine and gemcitabine (determined by MTT-assay) were selected for RNA isolation. We were unable to isolate a sufficient amount of RNA from 5 of these samples. Therefore, the study population consists of 50 newly diagnosed pediatric AML patients. Patient characteristics are listed in Table 2. This selected group of AML patients did not differ significantly regarding to age (p=0.30), white blood cell (WBC) count (p=0.14), sex (p=0.39), or *in vitro* sensitivity to ara-C (p=0.50) from a large group of AML samples that we have previously characterized for *in vitro* drug)[31] and was therefore considered to be representative.

Table 2. Patient characteristics.

Number, (n)	50
Sex (♂:♀), (%)	62 : 38
Age, yrs. (median; range)	10.7 (0.1 – 16.8)
WBC*, x10^9/L (median; range)	79.3 (2.1 – 524.0)
AML subtypes, (%)	
FAB M0	4
FAB M1	12
FAB M2	16
FAB M3	8
FAB M4	36
FAB M5	18
Unknown	6

*WBC: White blood cell count

mRNA expression levels of enzymes involved in the metabolism of deoxynucleoside analogs in AML and FAB-type subgroups

Using quantitative real-time PCR the mRNA expression levels of *hENT1, dCK, PN-I, CDA, dCMPD, RR1, RR2* and *CTPs* were determined. Measurable amounts of all 8 genes were found in all samples. Sample source (bone marrow (n=37) or peripheral

blood (n=13)) and the time interval between tissue acquisition and processing/storage of the cells (within 24 or 48 hrs) did not influence mRNA expression of the enzymes and all samples were therefore evaluated together in the following analyses. Genes were expressed with considerable variability between various patients (Figure 2). We investigated the association between all 8 genes and several diagnostic features. There was no difference in mRNA expression levels of target genes between boys and girls, nor was there a relation between the expression levels of these genes and initial WBC counts.

For the analysis with FAB-type, patients were divided into 3 subgroups: FAB M1/M2, FAB M4 and FAB M5. FAB M1 and M2 were taken together because they did not differ in age, sex, WBC, drug resistance or mRNA expression levels (data not shown). FAB M0 and FAB M3 were excluded because of the limited number of samples. FAB M5 expressed 1.8-fold (p=0.007) lower levels of *PN-I* compared to FAB M1/M2. We did not observe any other significant differences (Table 3).

Table 3. Relative mRNA expression of *hENT1* and enzymes involved in ara-C metabolism between AML FAB-type subgroups.

Gene	FAB M1/2 (n=14)	FAB M4 (n=18)	FAB M5 (n=9)
hENT1	0.68 (0.30 - 0.76)	0.51 (0.33 - 0.75)	0.57 (0.48 - 0.95)
dCK	0.61 (0.45 - 1.34)	0.52 (0.42 - 0.89)	0.55 (0.17 - 0.85)
CDA	0.069 (0.009 - 0.24)	0.14 (0.06 - 0.20)	0.16 (0.08 - 0.21)
dCMPD	1.09 (0.72 - 1.56)	0.89 (0.70 - 1.19)	0.60 (0.52 - 1.09)
PN-I	0.79 (0.51 - 1.37)	0.60 (0.39 - 0.85)	0.43 (0.16 - 0.50)*
RR1	0.18 (0.11 - 0.29)	0.15 (0.06 - 0.22)	0.15 (0.08 - 0.22)
RR2	0.05 (0.02 - 0.09)	0.04 (0.01 - 0.08)	0.05 (0.02 - 0.17)
CTPs	0.28 (0.20 - 0.32)	0.20 (0.14 - 0.33)	0.34 (0.17 - 0.47)

Indicated values are the median expression levels per group. Between parenthesis, the 25th-75th percentiles are given. * $p<0.01$ compared to FAB M1/2

In vitro deoxynucleoside analog cytotoxicity

Dose-response curves were obtained for all drugs and marked differences between individual patients were found. The median (25th-75th percentile) ara-C LC_{50} value was 1.70 μM (0.59 - 3.38 μM; n=50). For the purine analogs 2-CdA and F-ara-A group median LC_{50} values were 0.073 μM (0.051 - 0.098 μM; n=46) and 1.19 μM (0.66 - 2.27 μM; n=47), respectively. The group median for dFdC was 10.04 μM (2.05 - 20.86 μM; n=48), while DAC was only active in very high concentrations (median LC_{50} value = 3426 μM (717 - 5700 μM; n=48)).

Correlations between mRNA expression levels and *in vitro* sensitivity to deoxynucleoside analogs

hENT1 mRNA expression inversely correlated with the LC_{50} values of ara-C (r_p=-0.46; p=0.001; n=50) and also seemed to correlate inversely with the LC_{50} values of 2-CdA

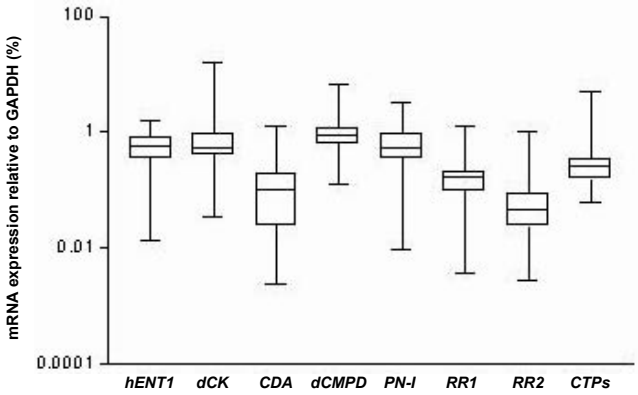

Figure 2. Expression levels of genes that potentially are involved in ara-C resistance.
Relative mRNA expression of the human equilibrative nucleoside transporter 1 (*hENT1*), deoxycytidine kinase (*dCK*), pyrimidine nucleotidase I (*PN-I*), cytidine deaminase (*CDA*), ribonucleotide reductase subunit 1 and 2 (*RR1* and *RR2*) and CTP synthetase (*CTPs*) were determined by quantitative real-time PCR (Taqman) analysis in 50 pediatric AML samples, obtained at diagnosis. Measurable amounts of all genes were found in all patients. Targets were expressed with great variability; group medians, 25th/75th percentiles and the ranges are given.

(r_p=-0.30; p=0.04, n=46), DAC (r_p=-0.29; p=0.04, n=48) and dFdC (r_p=-0.33; p=0.02, n=48). In other words, increased sensitivity to deoxynucleoside analogs was directly related to increased mRNA expression of the *hENT1* nucleoside transporter. Furthermore, decreased *dCK* mRNA expression seemed to correlate with resistance to dFdC (r_p=-0.31; p=0.03, n=48). Also, resistance to DAC seemed to correlate with increased *CDA* mRNA levels (r_p=0.33; p=0.03, n=48). The accumulation of ara-CTP could depend on the ratio of the activation enzyme dCK and the inactivation enzymes *PN-I* and *CDA*. Therefore, we also studied the relation between the *dCK/PN-I* and *dCK/CDA* ratios and *in vitro* drug sensitivity. The *dCK/PN-I* ratio correlated inversely with the LC_{50} values for dFdC (r_p=-0.45, p=0.001, n=47) and the *dCK/CDA* ratio seemed to correlate with the LC_{50} values for DAC (r_p=-0.29; 0.04, n=48). We did not observe correlations between these ratios and *in vitro* sensitivity to ara-C, 2-CdA and F-ara (Table 4).

mRNA expression levels of *hENT1, dCK, PN-I, CDA, dCMPD, RR1/2* and *CTPs* were entered into a stepwise multivariate regression model to identify the most important indicators with respect to *in vitro* sensitivity to deoxynucleoside analogs (dependent variables LC_{50} values ara-C, 2-CdA, DAC, F-ara-A or dFdC). In multivariate analysis, *hENT1* mRNA expression predicted *in vitro* sensitivity to ara-C (p=0.002). Furthermore, *CDA* mRNA expression levels seemed to predict *in vitro* sensitivity to DAC (p=0.02), while other factors did not reach

significance. Also when we divided the AML samples in three subgroups based on their *in vitro* ara-C sensitivity, resistant patients expressed 3-fold lower *hENT1* mRNA levels compared with sensitive patients (p=0.003; Figure 3).

Table 4. Correlation between expression levels of potential resistance factors and *in vitro* deoxynucleoside analog sensitivity (LC_{50} values) in 50 primary pediatric AML samples.

Resistance factor	Pearson correlation	Ara-C	2-CdA	DAC	F-ara-A	dFdC
hENT1	r_p	-0.46**	-0.30*	-0.29*	-0.24	-0.38*
	p-value	0.001	0.04	0.04	0.09	0.02
dCK	r_p	-0.11	-0.09	-0.04	0.004	-0.31*
	p-value	0.43	0.55	0.79	0.98	0.03
CDA	r_p	0.13	-0.09	0.33*	0.06	0.001
	p-value	0.38	0.57	0.02	0.69	0.99
dCK/PN-1	r_p	-0.27	-0.09	-0.16	-0.26	-0.45**
	p-value	0.06	0.55	0.29	0.08	0.001
dCK/CDA	r_p	-0.17	0.02	0.29*	-0.05	-0.18
	p-value	0.24	0.87	0.04	0.75	0.23

*Pearson correlation (r_p) significant at the 0.05 level (2-tailed).

**Pearson correlation (r_p) significant at the 0.01 level (2-tailed).

Correlations between the mRNA expression levels of *dCMPD, PN-I, RR1, RR2* and *CTPS,* and *in vitro* sensitivity to the different deoxynucleoside analogs, were statistically not significant.

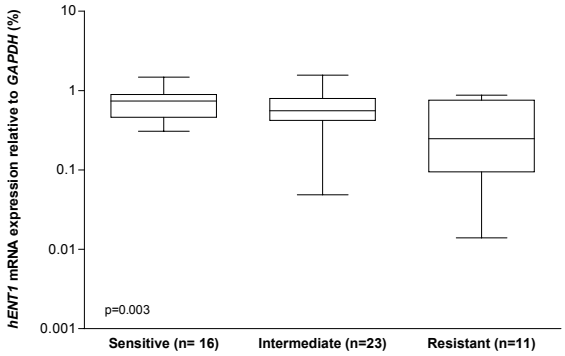

Figure 3. *hENT1* mRNA expression in relation to *in vitro* ara-C sensitivity.
AML patient samples were divided into three subgroups based on *in vitro* ara-C sensitivity (sensitive = LC_{50} <0.98 μM), intermediate = 0.98< LC_{50} <5.18 μM) and resistant = LC_{50} >5.18 μM). Patients that are resistant to ara-C *in vitro* expressed 3.0-fold lower *hENT1* mRNA levels compared with ara-C sensitive patients. P-value were determined by one-way Anova.

DISCUSSION

For AML, ara-C is the essential drug during induction and consolidation therapy and is given both at intermediate and high-dose schedules.[32,33] In the present study we analyzed possible resistance factors to ara-C, and other clinically important deoxynucleoside analogs, in AML by measuring the gene expression of the key players in transport and metabolism

of ara-C. Quantitative real-time PCR analysis revealed that AML FAB-M5 expressed lower levels of the ara-C degrading enzyme *PN-I* compared with FAB-M1/2. Although the analysis included only a limited number of samples, this finding may provide an explanation for the relative sensitivity to ara-C of AML FAB-M5 that we reported previously.[31]

We studied the relation between the mRNA expression level of potential ara-C resistance factors and *in vitro* sensitivity to deoxynucleoside analogs. Although *in vitro* drug resistance testing differs considerably from the *in vivo* situation, it does provide valuable indications as to which factors might be important in drug sensitivity *in vivo*.[34] *hENT1* mRNA expression correlated with sensitivity to ara-C and also seemed to correlate with sensitivity to 2-CdA, DAC and dFdC, indicating that transport across the cell membrane is an important step for deoxynucleoside analog induced cytotoxicity. In multivariate analysis *hENT1* mRNA expression was the most important factor determining sensitivity to ara-C. This might be explained by the fact that entry of ara-C into the cell is mainly dependent on hENT1-mediated transport.[5,6,35,36] In contrast, 2-CdA, DAC and dFdC differ from ara-C with respect to their preferential nucleoside transporters and can be transported across the cell membrane by other members of the nucleoside transporter family as well.[37] 2-CdA can enter cells via hENT1, hENT2 and the human concentrative nucleoside transporter 3 (hCNT3)[37,38], while hENT1, hENT2, hCNT1 and hCNT3 are able to mediate uptake of dFdC into cells.[37] hENT1 mediated influx, however, seems to be a pivotal factor in ara-C cytotoxicity. Patients resistant to ara-C *in vitro* expressed 3-fold lower *hENT1* mRNA levels. Our results are supported by the fact that hENT1 has been implicated as a crucial factor in ara-C sensitivity in previous studies.[39,40] Galmarini *et al* measured *hENT1* mRNA expression in adult AML samples and demonstrated that *hENT1* deficiency was related to a shorter disease free survival.[40] In addition, elevated *hENT1* mRNA expression explained the remarkable ara-C sensitivity of infants with *MLL* gene rearranged acute lymphoblastic leukemia (ALL).[25] *hENT1* may therefore be a valuable predictor of ara-C sensitivity at diagnosis. Unfortunately we were not able to asses the relation between *hENT1* expression and *in vivo* response to treatment due to the heterogenity of the treatment and the limited follow-up time. Patients were treated according to different treatment protocols and several different dosing schedules. AML patients may, however, benefit from screening for *hENT1* mRNA levels at diagnosis, because of its significance for ara-C dosing. At intermediate dose ara-C (100-200 mg/m^2) plasma levels are in the μM range and transport across the cell membrane is solely dependent on nucleoside transporters.[41] At high dose ara-C (1-3 g/m^2) however, hENT1 seems less crucial, although plasma concentrations might not exceed the Km of the transporter-mediated influx. At these high concentrations, Ara-C also enters the cell by passive diffusion, while dCK will be saturated.[42,43] Patients with a low *hENT1* mRNA level could potentially benefit from up-front high dose ara-C treatment or an ara-C analog that is not dependent on transporter-mediated influx. A compound such as troxacitabine for example, which

passively diffuses across the cell membrane due to its unusual L-configuration[44,45], might be able to circumvent ara-C resistance caused by low *hENT1* expression.

Most studies on ara-C resistance have focused on the conversion of ara-C to ara-CTP and several studies have linked reduced *dCK* mRNA expression or functional activity to ara-C resistance.[46-49] In acute leukemia, relapsed ALL and AML patients have been shown to express decreased *dCK* mRNA levels.[50] In contrast, dCK was not rate-limiting in the formation of ara-CTP in infants with *MLL* gene rearranged ALL.[25] In this present study we did not observe a correlation between *dCK* mRNA expression and *in vitro* ara-C sensitivity in AML blasts. Although there was a considerable range in *dCK* mRNA levels in AML blasts, the median expression was quite high, and it therefore seems unlikely that low *dCK* expression plays a role in ara-C resistance in childhood AML at diagnosis. We have previously reported on *dCK* mRNA levels in childhood AML blasts and most AML samples expressed mRNA levels that were in the range of cell lines that are sensitive to ara-C.[51] Nevertheless, reduced *dCK* mRNA expression may be involved in resistance to gemcitabine. Both *dCK* mRNA and dCK protein levels have been shown to predict *in vivo* gemcitabine sensitivity.[52] Our present study also indicated that reduced *dCK* mRNA expression could contribute to *in vitro* gemcitabine resistance in AML blasts. It should be mentioned however that the Pearson correlation coefficient was low and was not significant in multivariate analysis.

Finally, multivariate analysis showed that DAC resistance seemed to correlate with increased mRNA levels of the inactivating enzyme CDA. DAC was initially developed as a cytotoxic agent and has activity in several hematological malignancies. Low-dose DAC is currently enjoying a revival as a specific inhibitor of DNA hypermethylation in cancer.[20] DAC is an excellent substrate for CDA and elevated *CDA* mRNA levels may contribute to resistance to DAC.[53]

In conclusion, our findings indicate that reduced drug influx into the cell caused by decreased *hENT1* mRNA expression might be involved in resistance to ara-C, and other deoxynucleoside analogs, in childhood AML.

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Chapter 6

Multidrug resistance genes in infant Acute Lymphoblastic Leukemia;

Ara-C is not a substrate for the Breast Cancer Resistance Protein (BCRP)

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ABSTRACT

Infants with acute lymphoblastic leukemia (ALL) are more resistant to chemotherapeutic drugs than older children with ALL, except for Ara-C. The mechanisms underlying drug resistance in infant ALL, however, remain unknown. Possibly, multidrug resistance (MDR) proteins like P-glycoprotein, multidrug resistance associated protein (MRP1), lung resistance-related protein (LRP/MVP), and the breast cancer resistance protein (BCRP), play a role. Accordingly we determined the mRNA levels of the genes encoding these proteins in infants (n=13) and older children (non-infants) (n=13) with ALL, using quantitative real-time PCR. Infant ALL patients expressed 2.4-fold less *BCRP* mRNA (p=0.009) than non-infants with ALL. *MDR*1, *MRP*1 and *LRP*/*MVP* expression did not differ significantly between both patient groups. The expression of the MDR genes did not correlate to prednisolone, vincristine, daunorubicin or Ara-C cytotoxicity, with the exception of *BCRP* expression, which significantly correlated with resistance to Ara-C (r_s=0.53, p=0.012), suggesting that Ara-C might be a substrate for BCRP. However, culturing patients ALL cells in the presence of the BCRP inhibitor Ko143 had no effect on Ara-C sensitivity. Moreover, inhibiting Bcrp1 in the *Mdr1a*, *Mdr1b* and *Mrp1* deficient and Bcrp1 over-expressing mouse cell line Mef3.8/T6400, also did not modulate Ara-C cytotoxicity. Therefore we conclude that Ara-C is not a substrate for BCRP and that MDR proteins do not play a significant role in drug resistance in infant ALL.

INTRODUCTION

Nowadays, approximately 70% of the children diagnosed with acute lymphoblastic leukemia (ALL), treated using combination chemotherapy, achieve and remain in continuous complete remission. For a minority of patients with ALL, however, the prognosis is far less promising. Infants (i.e. children <1 year of age) form the most striking example of a subgroup of ALL patients who have failed to benefit from the greatly improved treatment regimens developed over the last few decades. The prognosis for infants with ALL still is very poor, with an event-free survival (EFS) of approximately 35%.[1] Treatment failure in infant ALL patients seems to be associated with cellular drug resistance. Pieters *et al*.[2] demonstrated that leukemic cells from infants with ALL are *in vitro* significantly more resistant to chemotherapeutic drugs, especially to prednisone and L-asparaginase, compared with cells from older children with ALL. One exception, however, is Ara-C to which infant ALL cells are highly sensitive.[2]

Multidrug resistance (MDR) is described as (cross)resistance to structurally unrelated cytotoxic drugs, which severely limits the effectiveness of the chemotherapeutic treatment of the patient. Since leukemic cells from infants with ALL are *in vitro* resistant to multiple chemotherapeutic drugs, infant ALL patients can legitimately be classified as multidrug resistant. MDR has been associated with decreased cellular drug retention as a result of increased drug efflux mediated by specialized ATP-dependent transmembrane transporter proteins. Several MDR related drug efflux proteins have been characterized, most of which appeared to be members of the ATP-binding cassette (ABC) transporter superfamily. The most extensively studied multidrug resistance protein is the Permeability-glycoprotein (P-gp) encoded by the *MDR*1 gene. P-gp has been described to have broad substrate specificity, decreasing the intracellular retention of, among others, anthracyclins (e.g. daunorubicin, doxorubicin), anthracenes (e.g. mitoxantrone), vinca alkaloids (e.g. vincristine, vinblastine) and epipodophyllotoxins (etoposide and teniposide).[3] Other identified MDR proteins are the multidrug resistance related protein 1 (MRP1),[4] lung resistance-related protein/major vault protein (LRP/MVP)[5], and the recently discovered breast cancer resistance protein (BCRP).[6,7] MRP1 is a member of the MRP family of ABC transporters, of which to date eight members have been identified. So far, MRP1 is the only MRP family member that has been associated with clinical multidrug resistance. Although MRP1 shares only 15% homology with P-gp, the phenotype associated with MRP1 overexpression results in a MDR phenotype comparable to that of P-gp over-expressing cells.[8] Lying on a separate limb of the phylogenetic tree,[9] BCRP is evolutionarily distinct from the other ABC transporters, and its gene encodes a so-called half-transporter that dimerizes to form an active transporter.[10] This may suggest a separate role for BCRP in clinical drug resistance. To date, accepted substrates for BCRP include camptothecins, mitoxantrone and related molecules[11], and methotrexate.[12] LRP was identified as the major vault pro-

tein,[13] an important component of well conserved cellular organelles called vaults, which are up-regulated in multidrug resistant cancer cell lines.[14] Unlike P-gp, MRP1 and BCRP, LRP/MVP is not a member of the ABC transporter family. It is thought that LRP/MVP decreases the effectiveness of cytotoxic drugs, either by regulating nucleus-cytoplasmatic transport of cytotoxic drugs away from the nucleus and/or by involvement in sequestration of cytotoxic drugs in exocytotic vesicles.[13] LRP/MVP has been reported to be involved in resistance to vincristine, doxorubicin and etoposide.[15]

The exact clinical value of the above described multidrug resistance proteins in childhood ALL is not clear and existing data are conflicting.[16] Moreover, to date no data exists on the possible role these MDR efflux proteins might play in the observed drug resistance in infant ALL patients. Therefore, we determined and compared the expression levels of *MDR1*, *MRP1*, *LRP/MVP* and *BCRP* using quantitative real-time PCR (Taqman) analysis, in leukemic samples form infants and non-infants diagnosed with ALL. In addition, the obtained mRNA expression levels of these MDR genes were correlated to the level of cytotoxicity induced in these samples by several important chemotherapeutic drugs used in the treatment of childhood ALL.

MATERIALS AND METHODS

Patient samples

Bone marrow and/or peripheral blood samples of 13 untreated infants (<1 year of age) initially diagnosed with ALL were collected at the Erasmus MC - Sophia Children's Hospital, and other hospitals participating in the INTERFANT-99 treatment protocol. Samples of 13 initially diagnosed ALL patients older than 1 year of age were obtained from the German COALL study group (Prof. Dr. G. E. Janka-Schaub, Hamburg, Germany). The median age in the infant and non-infant group was 0.5 and 6.0 years respectively. The immunophenotype of the infants predominantly was pro-B and pre-B, whereas most of the non-infant ALL patients had a common ALL (c-ALL) immunophenotype. In the infant group, 77% of the patients carried a rearrangement of the *MLL* gene on chromosome 11q23. In contrast, all the non-infant ALL patients harbored germ line *MLL* genes.

Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/ml; Nycomed Pharma, Oslo, Norway), centrifuged at 480 *g* for 15 minutes at room temperature. The collected mononuclear cells were washed twice and kept in culture medium consisting of RPMI 1640 medium (Dutch modification without L-glutamine; Gibco BRL, Life Technologies), 20% fetal calf serum (FCS ; Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Gibco BRL, Life Technologies, Breda, The Netherlands) 5 μg/ml insulin, 5 μg/ml transferrin,

5 ng/ml sodium selenite (ITS media supplement; Sigma, St Louis MO, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.125 μg/ml fungizone (Gibco BRL, Life Technologies) and 0.2 mg/ml gentamycin (Gibco BRL, Life Technologies). Contaminating non-leukemic cells were removed using immunomagnetic beads (Dynal ASA, Oslo, Norway). All samples contained ≥90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from a minimum of 5 x10^6 cells using TRIzol reagent (Gibco BRL, Life Technologies) according to the manufacturer's protocol, except for minor modifications; An additional phenol-chloroform extraction was performed and the isopropanol precipitation at –20°C was facilitated by adding 1 μl (20 μg/ml) glycogen (Roche, Almere, The Netherlands). After precipitation with isopropanol, RNA pellets were dissolved in 20 μl RNAse-free TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0). The RNA was quantified spectrophotometrically. Following a denaturation step of 5 minutes at 70°C, 1 μg of RNA was reverse transcribed to single stranded cDNA using a mix of random hexamers (2.5 μM) and oligo dT primers (20 nM). The RT reaction was performed in a total volume of 25 μl containing 0.2 mM of each dNTP (Amersham Pharmacia Biotech, Piscataway NJ, USA), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison Wisconsin, USA) and 25 U RNAsin (Promega), at 37°C for 30 minutes, 42°C for 15 minutes and 94°C for 5 minutes. The obtained cDNA was diluted to a final concentration of 8 ng/μl.

Quantitative real-time PCR (Taqman technology)

The mRNA expression levels of *MDR*1, *MRP*1, *LRP/MVP* and *BCRP* and the endogenous housekeeping gene *GAPDH* as a reference, were quantified using real-time PCR analysis (Taqman) essentially as described recently.[17] On an ABI Prism 7700 sequence detection system (PE Applied Biosystems), specific PCR products were amplified and detected using dual-fluorescent non-extendable probes labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) at the 5'-end and 3'-end, respectively. Primers and probe combinations, reported previously,[17] were designed using OLIGO 6.0 primer analysis software (Medprobe, Olso, Norway). All PCRs showed comparable efficiencies of E ≥ 95%, and were performed in duplicate in a reaction volume of 50 μl containing 1x Taqman buffer A (PE Applied Biosystems), 4 mM $MgCl_2$, 200 μM of each dNTP (Amersham Pharmacia Biotech), 300 nM forward and reverse primer, 200 nM probe, 1.25 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems) and 40 ng of cDNA from each patient as a template. Samples were heated for 10 minutes at 95 °C and amplified for 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. A serial dilution of cDNA derived from a cell line RNA-pool (CEM, K562, and two EBV transformed

lymphoblastoid B-cell lines) was amplified in parallel as a control to verify amplification efficiency within each experiment. For each patient, the relative mRNA expression levels of *MDR*1, *MRP*1, *LRP/MVP* and *BCRP* were calculated using the comparative cycle time (C_t) method.[18] Briefly, the target PCR C_t values, i.e. the cycle number at which emitted fluorescence exceeds 10x the standard deviation of base-line emissions as measured from cycles 3 to 15, is normalized to the *GAPDH* PCR C_t value by subtracting the *GAPDH* C_t value from the target PCR C_t value. The relative mRNA expression level to GAPDH for each target PCR can be calculated using the following equation :

$$\text{Relative mRNA expression} = 2^{-[\text{Ct target} - \text{Ct GAPDH}]} \times 100\%.$$

In vitro drug cytotoxicity assay (MTT assay)

In vitro drug cytotoxicity was determined using the MTT assay as described by Pieters *et al.*[19] Briefly, 100 µl aliquots of cell suspension (1.6 x10^5 cells) were cultured in round-bottomed 96-well microtitre plates (Greiner bio-one) in the presence of six different concentrations of each drug in duplicate. The drugs tested were: prednisone (PRED, Bufa, Uitgeest, The Netherlands), vincristine (VCR, Teva Pharma, Mijdrecht, The Netherlands), daunorubicin (DNR, Rhône-Poulenc-Rorer, Amstelveen, The Netherlands), and Ara-C (Cytosar, Pharmacia & Upjohn BV, Woerden, The Netherlands). The range of end concentrations and the dilution factors were: PRED (0.0076-250 µg/ml, 8-fold), VCR (0.049-50 µg/ml, 4-fold), DNR (0.0019-2 µg/ml, 4-fold) and Ara-C (0097-10 µg/ml, 4-fold). Control cells were cultured in eight wells in the absence of drugs. 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 Aldrich, Zwijndrecht, The Netherlands) was added, and the plates were incubated for an additional six hours under the same conditions. During this final 6-hour incubation, the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals by viable cells only. The formazan crystals were dissolved by adding 100 µl acidified isopropanol (0.04 N HCl-isopropyl alcohol) and the optical density (OD), which is linearly related to the number of viable cells,[20] was measured spectrophotometrically at 562 nm. After subtraction of the blank values, the leukemic cell survival (LCS) was calculated by the equation :

$$\text{LCS} = (\text{OD}_{\text{Day 4}} \text{ treated well} / \text{mean OD}_{\text{Day 4}} \text{ control wells}) \times 100\%$$

Drug sensitivity was assessed by the LC_{50}, the drug concentration lethal to 50% of the cells. Evaluable assay results were obtained when a minimum of 70% leukemic cells was present in the control wells after 4 days of incubation and when the control optical density (OD) was ≥ 0.050.[19]

To determine Ara-C cytotoxicity in the absence and presence of the specific BCRP inhibitor Ko143,[21] leukemic cells from patients were pre-incubated with 200 nM Ko143 or with an equal volume of PBS for 1 hour at 37°C in humidified air containing 5% CO_2. Without washing the cells or removing the inhibitor from the culture medium, Ara-C cytotoxicity was determined using the MTT-assay as described above.

Cytotoxicity assays in the Mef3.8 and Mef3.8/T6400 cell lines

Both the Mef3.8 and the Mef3.8/T6400 cell lines were maintained in DMEM supplemented with 10% FCS (HyClone), 10 mM HEPES, penicillin and streptomycin. Mef3.8 cells are adherent mouse embryo fibroblasts which are nullizygous for P-gp and Mrp1.[22] The Mef3.8/T6400 subline was obtained by selection for resistance to topotecan and expresses high levels of wild-type Bcrp1.[22,23] The subline was routinely maintained under continuous selection with 6.4 μM topotecan, but this concentration was reduced to 0.64 μM for 2 days prior to use of these cells in the cytotoxicity assay.

Sub-confluent cells were seeded in 96-well pates at a density of 500 cells/well in a volume of 100 μl. After allowing attachment of the cells for 4 hours, the cells were exposed to concentration series of Ara-C or mitoxantrone, in the presence or absence of 200 nM of the specific Bcrp1 inhibitor Ko143. After 4-5 days, while cells were still sub-confluent in the untreated wells, the wells were stained with 1:4000 Sybr Green I (Molecular Probes, Eugene, OR) nucleic acid stain, diluted in a hypotonic lysis buffer (10 mM Tris, pH8.0, 2.5 mM EDTA, 0.1% Triton X-100) using 200 μl/well, and equilibration was allowed for 24 hours at 4°C in the dark. Relative cell proliferation was quantified by measuring Sybr Green I fluorescence using a plate reader (Cytofluor 4000 ; PerSpective Biosystems, Framingham, MA) with 485 nm excitation and 530 nm emission filters. Cytotoxicity of the drugs was expressed as the IC_{50} value, i.e. the concentrations of drugs which inhibits 50% of the growth of the cells.

RESULTS

Using quantitative real-time PCR (Taqman technology), the mRNA expression levels of *MDR*1, *MRP*1, *LRP/MVP* and *BCRP* were determined in leukemic cells from infants (n=13) and older children (non-infants) (n=13) with newly diagnosed ALL. Infants expressed significantly less *BCRP* mRNA (p=0.009) compared to older children with ALL. The difference in the median relative *BCRP* mRNA expression between the infant and non-infant group was 2.4-fold. No significant differences in mRNA expression of *MDR*1, *MRP*1 and *LRP/MVP* were found, although a trend towards decreased *MRP*1 mRNA expression in infants was observed (Figure 1).

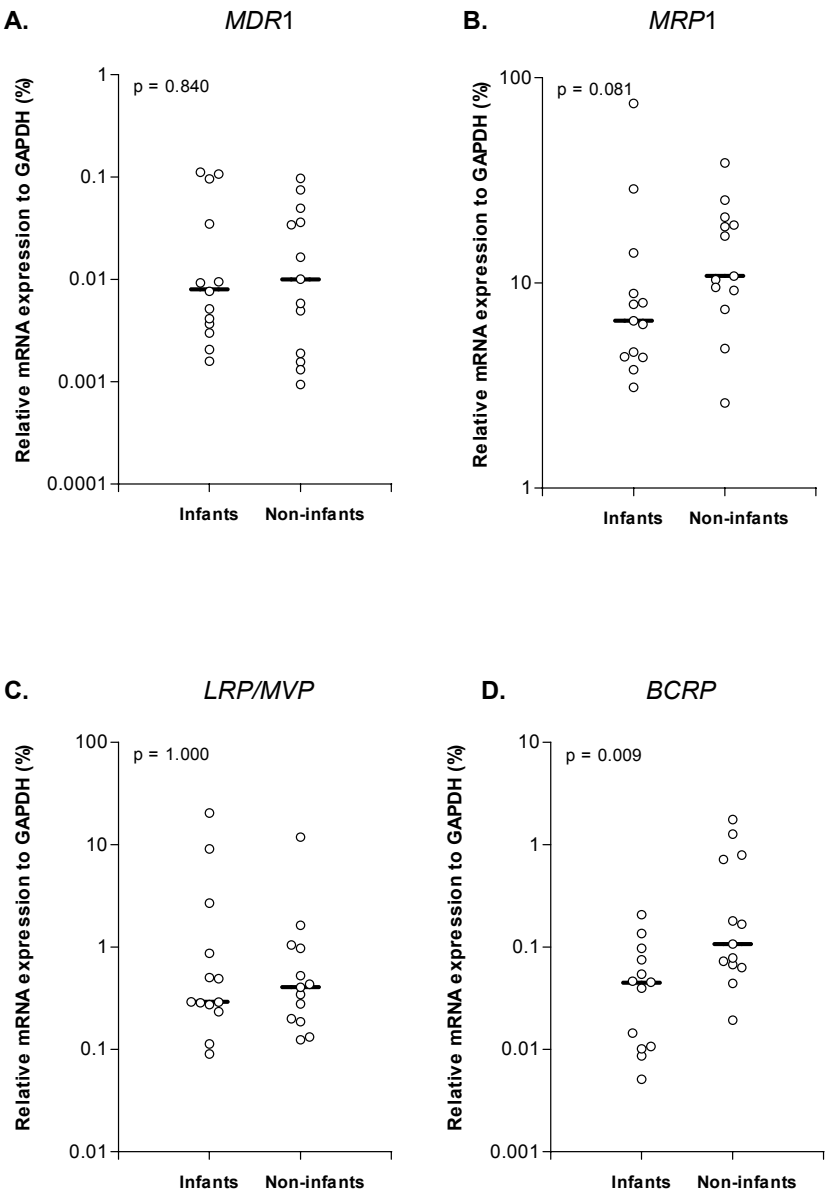

Figure 1. Relative mRNA expression of *MDR1*, *MRP1*, *LRP/MVP* and *BCRP* in primary infant and non-infant ALL samples.

Differences in *MDR*1, *MRP*1, *LRP/MVP* and *BCRP* mRNA expression between infants (n=13) and non-infants (n=13) with ALL were analyzed using the Mann-Whitney U test. The lines indicate the median values, circles (o) represent individual patients.

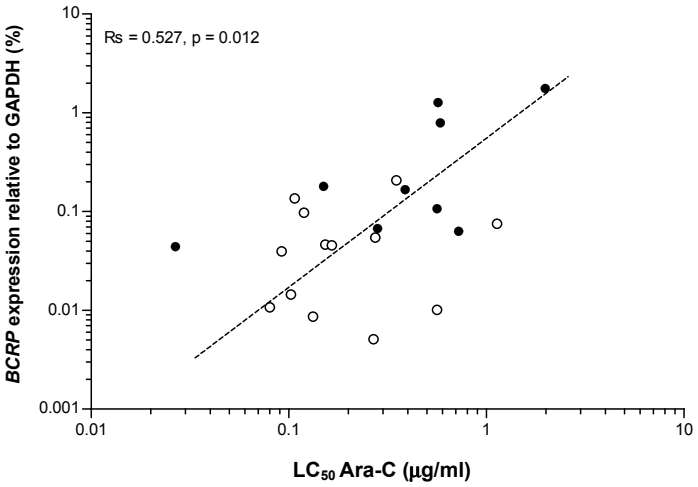

Figure 2. Relation between Ara-C cytotoxicity and *BCRP* mRNA expression in pediatric ALL.
The correlation between the Ara-C cytotoxicity (LC_{50} in µg/ml) and the relative expression of *BCRP* mRNA was calculated using the Spearman's rank correlation test. Open circles (o) indicate infants with ALL and closed circles (•) non-infant ALL patients.

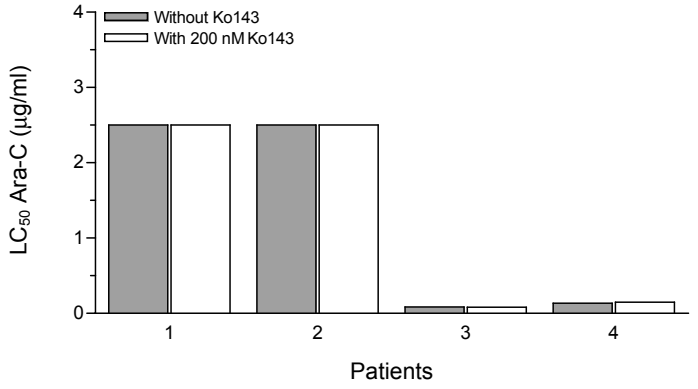

Figure 3. Ara-C cytotoxicity in the absence and presence of Ko143 in pediatric ALL.
Ara-C cytotoxicity determined in two Ara-C resistant and two Ara-C sensitive patients with ALL in the absence and presence of 200 nM of the specific BCRP inhibitor Ko143.

The obtained mRNA levels of the MDR genes were correlated to the cytotoxicity of several important chemotherapeutic drugs used in the treatment of childhood ALL, like prednisone, vincristine, daunorubicin and Ara-C. No positive correlation was observed between the mRNA expression of *MDR*1, *MRP*1, *BCRP* or *LRP/MVP* and the

cytotoxicity of the drugs tested, except for *BCRP* and Ara-C. *BCRP* mRNA expression positively correlated with the LC_{50} values of Ara-C (R_s=0.53, p=0.012) (Figure 2). These findings suggest that Ara-C might be a substrate for BCRP, and that Ara-C sensitivity in infant ALL may be explained by a decreased efflux of Ara-C out of these cells, mediated by BCRP. To test this hypothesis, Ara-C cytotoxicity in the leukemic cells from two Ara-C resistant and two Ara-C sensitive ALL patients was determined both in the absence and presence of 200 nM of the specific BCRP inhibitor Ko143. Inhibition of BCRP by Ko143, however, had no effect on Ara-C cytotoxicity (Figure 3).

Additionally, cytotoxicity to Ara-C and mitoxantrone was determined in cells from the *Mdr1a/b*$^{-/-}$, *Mrp1*$^{-/-}$ mouse embryo fibroblast cell line Mef3.8 and the Bcrp1 (the mouse homologue of human BCRP) over-expressing subline Mef3.8/T6400, both in the presence and absence of 200 nM of Ko143 (Table 1). Although the Bcrp1 over-expressing subline Mef3.8/T6400, derived by selection for topotecan resistance, appeared to be significantly more resistant to Ara-C compared to the parental Mef3.8 cell line ($p<0.05$), inhibition of Bcrp1 by Ko143 did not alter Ara-C cytotoxicity. Mitoxantrone, a known substrate for BCRP, served as a positive control in these experiments. Ko143 markedly sensitized the Mef3.8/T6400 cell line 68-fold to mitoxantrone ($p<0.001$) (Table 1).

Table 1. Bcrp1-mediated resistance to Ara-C in mouse Mef3.8/T6400 cells.

	IC50 (nM)		
	Mef3.8	**Mef3.8/T6400**	*Resistance Factor**
Ara-C	109 ± 50	184 ± 104	*1.6 ± 0.3 (p<0.05)*
Ara-C + 200 nM Ko143	117 ± 55	191 ± 106	*1.6 ± 0.7 (ns)*
*Fold sensitisation by Ko143**	*0.94 ± 0.11*	*0.98 ± 0.13*	
	(ns)	*(ns)*	
Mitoxantrone	0.66 ± 0.33	31 ± 11	*50 ± 8 (p<0.01)*
Mitoxantrone + 200 nM Ko143	0.52 ± 0.30	0.48 ± 0.20	*1.0 ± 0.2 (ns)*
*Fold sensitisation by Ko143**	*1.3 ± 0.2*	*68 ± 11*	
	(p<0.05)	*(p<0.01)*	

*Resistance and sensitization factors were calculated within each experiment. Means and standard deviations were derived from four independent assays. Results of two-tailed t-tests of the hypotheses that resistance factors and fold sensitization by Ko143 differ from 1.0 are shown in parentheses; *ns* = not significant.

DISCUSSION

Multidrug resistance is attributed to the over-expression of certain efflux proteins, that are capable of trafficking chemotherapeutic drugs out of the cell, like P-glycoprotein, MRP1 and BCRP, or away from the nucleus by LRP/MVP. The exact clinical value of these MDR proteins in childhood ALL, however, is not clear and existing data are

conflicting.[16] Increased levels of P-glycoprotein expression, both at the protein[24,25] and mRNA level[26], as well as increased P-glycoprotein functioning[27,28] have been shown to be significant unfavorable prognostic factors for clinical outcome in childhood ALL. Other studies, however, contradict this finding and show that P-glycoprotein expression has no clinical value.[29,30,31] Data on the prognostic significance of the other MDR proteins in childhood ALL are scarce. One study on a group of Indian ALL patients, most of whom were children, reported significantly higher *MRP*1 mRNA expression at relapse than at presentation or remission.[32] However, this finding has not been confirmed by others,[33,34] and MRP1 measured at the protein level appeared to have no prognostic importance[30] in yet another study. *LRP/MVP* mRNA expression has been reported not to be of prognostic value in newly diagnosed childhood ALL.[33] Furthermore, no differences in LRP/MVP protein expression have been reported between initial and relapse ALL samples,[30] only in multiple relapse samples, LRP/MVP expression was found to be significantly increased.[30] Even so, it has been suggested that children with initial ALL and no detectable LRP/MVP expression experience significantly longer relapse-free intervals compared to patients with LRP expression.[35] Additional studies from our laboratory on a group of 146 uniformly treated children with ALL showed that neither P-gp, MRP1 nor LRP/MVP expression, measured at initial diagnosis, correlated with clinical outcome after combination chemotherapy (unpublished data). To date, only a single study on BCRP expression in childhood ALL has been reported, in which *BCRP* mRNA was measured in 47 initial and 20 relapsed ALL samples. No differences between initial and relapsed ALL were observed and *BCRP* mRNA expression did not seem to have any prognostic significance.[36]

There is no published data either on the role the MDR proteins play in infant ALL or on possible differences in the expression of the MDR genes between infants and older children with ALL. However, since infants with ALL are more resistant to chemotherapeutic drugs than older children,[2] such data may well contribute to our understanding of the mechanisms underlying drug resistance in infant ALL, which to date remain unknown. Hence, we measured the mRNA levels of *MDR*1, *MRP*1, *LRP/MVP* and *BCRP* in infant and non-infant ALL samples using quantitative real-time PCR. The only significant difference observed between both groups was that infants expressed 2.4-fold less *BCRP* mRNA. Furthermore, the only significant correlation found between the mRNA expression of the four MDR proteins and the cytotoxicity of several important chemotherapeutic drugs was between *BCRP* and Ara-C. The observed relationship indicated that patients with increased mRNA levels of *BCRP* are likely to be more resistant to Ara-C compared to children with lower *BCRP* mRNA levels, suggesting that Ara-C might be a substrate for BCRP. Nevertheless, culturing leukemic cells form ALL patients in the presence of Ko143, a specific BCRP inhibitor,[21] did not sensitize the leukemic cells to Ara-C. The finding that Ara-C was not a substrate for BCRP was

confirmed by additional cell line studies. Given the profound similarities between human BCRP and its mouse cognate Bcrp1,[23] the P-gp and Mrp1 deficient mouse embryo fibroblast cell line Mef3.8 and its Bcrp1 over-expressing sub line Mef3.8/T6400[22] constitutes a sound model for studying BCRP. Inhibition of Bcrp1 in these cell lines did not modulate Ara-C cytotoxicity, whereas both cell lines were markedly sensitized to mitoxantrone, a known substrate for BCRP.[11] In the Mef3.8/T6400 subline, 200 nM of Ko143 completely reversed the high level of resistance to mitoxantrone, demonstrating that culturing cells in the presence of this concentration of Ko143 indeed thoroughly inhibits Bcrp1.

Taken together our data suggest that, despite of the observed correlation between *BCRP* mRNA expression and Ara-C resistance, Ara-C is not a substrate for BCRP. Moreover, since leukemic cells from infants with ALL are more resistant to chemotherapeutic drugs compared with cells from older children with ALL,[2] but do not show increased expression of the MDR genes tested, we conclude that multidrug resistance proteins are unlikely to play a significant role in clinical drug resistance in infants with ALL.

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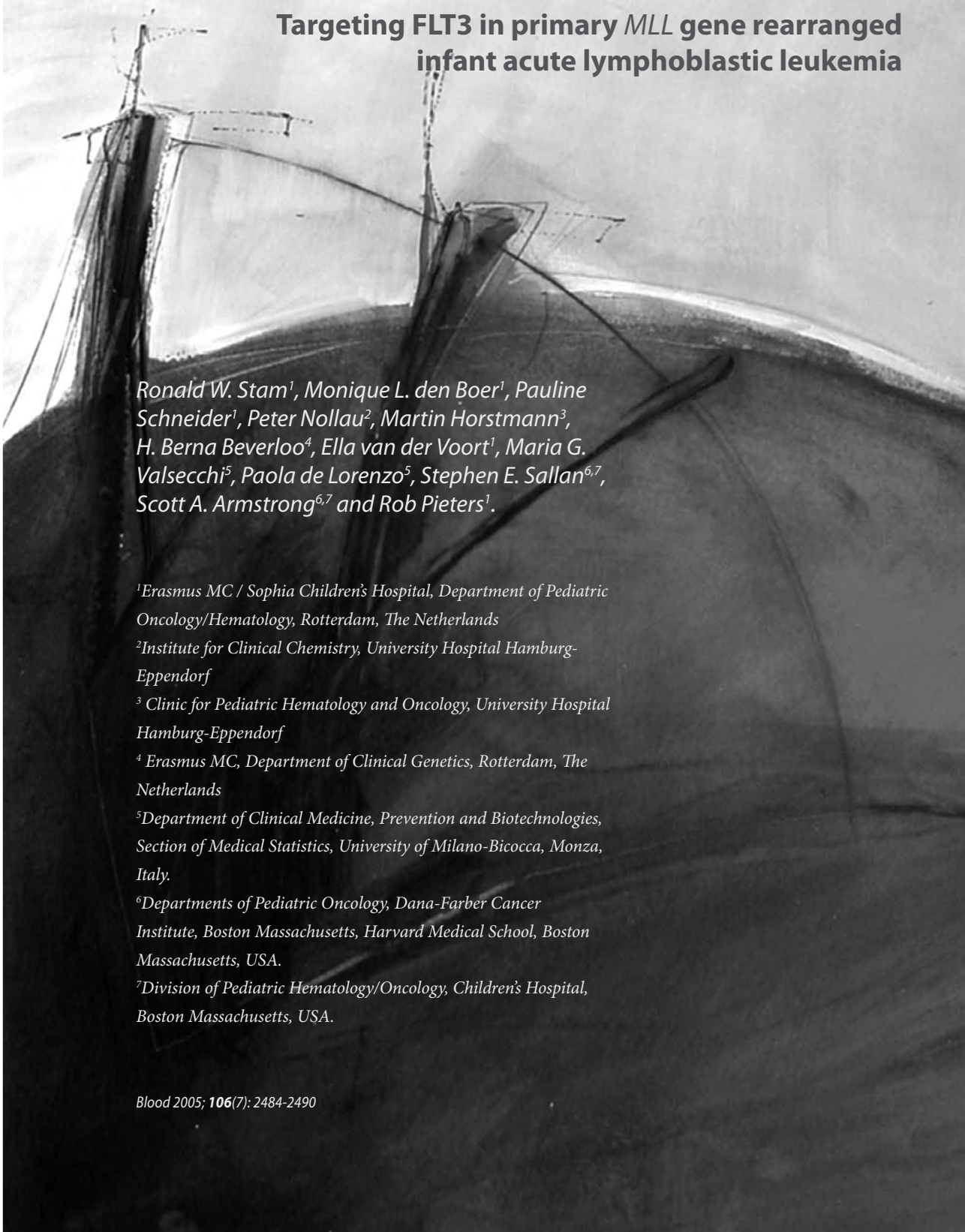
Chapter 7

Targeting FLT3 in primary *MLL* gene rearranged infant acute lymphoblastic leukemia



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ABSTRACT

Acute lymphoblastic leukemia (ALL) in infants is characterized by rearrangements of the *MLL* gene, drug resistance and a poor treatment outcome. Therefore novel therapeutic strategies are needed to improve prognosis. Recently we showed that *FLT3* is highly expressed in *MLL* rearranged ALL (MLL). Here we demonstrate *FLT3* expression in infant MLL patients (n=41) to be significantly higher compared to both infant (n=8) ($p<0.001$) and non-infant ALL patients (n=23) ($p=0.001$) carrying germ line *MLL* genes. Furthermore, leukemic cells from infant MLL patients were significantly more sensitive to the FLT3 inhibitor PKC412 than non-infant ALL cells, and at least as sensitive as ITD positive AML cells (MTT-assay). Surprisingly, activation loop mutations only occurred in ~3% (1/36) of the cases, and no FLT3/ITDs were observed. However, measuring FLT3 phosphorylation in infant MLL patients expressing varying levels of wild-type *FLT3* revealed that high-level *FLT3* expression is associated with ligand-independent FLT3 activation. This suggests that infant MLL cells displaying activated FLT3 as a result of over-expression can be targeted by FLT3 inhibitors like PKC412. However, at concentrations of PKC412 minimally required to fully inhibit FLT3 phosphorylation, the cytotoxic effects were only fractional. Thus, PKC412 induced apoptosis in infant MLL cells is unlikely to be a consequence of FLT3 inhibition alone, but may involve inhibition of multiple other kinases by this drug.

INTRODUCTION

Translocations involving the *Mixed Lineage Leukemia* (*MLL*, *HRX*, or *ALL1*) gene on chromosome band 11q23 are most frequently found in acute lymphoblastic leukemia (ALL) in infants (less than 1 year of age), with an incidence as high as ~80%.[1,2] The presence of *MLL* gene rearrangements is an independent prognostic factor for an adverse outcome.[2-5] Hence the prognosis for infant ALL is exceedingly poor with an event-free survival (EFS) of approximately 35%.[6] This poor treatment outcome is largely due to cellular drug resistance. Leukemic cells from infants with ALL are significantly more resistant to most chemotherapeutic drugs both *in vitro* and *in vivo*, as compared to cells from older children with ALL.[7-9] This is especially true for prednisone and L-asparaginase. Since the clinical outcome for infant ALL patients bearing germ line *MLL* genes appears to be much more favorable,[9-11] novel therapeutic targets specific for *MLL* rearranged infant ALL (infant MLL) are urgently needed.

In search for suitable targets, we recently compared gene expression profiles from *MLL* rearranged ALL patients with profiles from both ALL and acute myeloid leukemia (AML) patients carrying germ line *MLL* genes. This study demonstrated that *MLL* gene rearranged ALL is characterized by high-level expression of the gene encoding Fms-like tyrosine kinase 3 (*FLT3*, *STK-1*, *FLK-2*, or CD135).[12] FLT3 is a membrane-bound receptor for the hematopoietic growth factor FLT3 ligand (FLT3L or FL), and is important in early hematopoietic development.[13] Upon binding of FLT3L, wild-type FLT3 receptors dimerize and become activated by phosphorylation, positively affecting several signal transduction pathways all of which favor cell survival and proliferation.[14] In the absence of ligand binding, FLT3 only has minimal kinase activity as a consequence of auto-inhibition by the juxtamembrane (JM) domain of the receptor.[15]

With an incidence of approximately 30%, *FLT3* is the most frequently mutated gene in AML.[16] Mutations in *FLT3* appear to activate the receptor in a ligand-independent manner, constitutively promoting proliferation and survival, and thus providing the leukemic cell with a growth advantage and transforming capacity. Distinct types of such activating mutations within two separate regions of the *FLT3* gene have been described. The first are in-frame internal tandem duplications (ITDs) within the JM domain-coding sequence of *FLT3*.[17] These FLT3/ITDs disrupt the auto-inhibitory activity of the JM domain, leading to receptor dimerization and subsequent auto-phosphorylation in the absence of FLT3L.[18] The second type of activating mutations affect either Asp835 or Ile836 within the second tyrosine kinase domain of the FLT3 receptor. At first point mutations were described that resulted in alternative amino acids at Asp835.[19] Recently, insertions after Asp835, as well as deletions of the adjacent codon Ile836 (Δ836) have been reported.[20,21] Analogous to point mutations at Asp816 within a corresponding domain of the receptor tyrosine kinase c-KIT,[22] these mutations alter the conformation

of the activation loop from an inactive to an active state, allowing auto-phosphorylation, and thus activation of FLT3, again in the absence of its ligand.[14]

The identification of activating mutations and their high incidence in AML patients have led to the development of several small molecule inhibitors in order to selectively target the constitutive FLT3 signal, inducing leukemic cells to undergo programmed cell death (apoptosis). The potential of several FLT3 inhibitors as therapeutic drugs has been, or is currently being tested in phase I/II clinical trials in adults with relapsed or refractory AML or myelodysplastic syndromes (MDS).[23-27] Recently we found the staurosporine derivative PKC412, a known inhibitor of FLT3,[28] to be cytotoxic to acute lymphoblastic leukemia cell lines carrying translocations involving *MLL* and activated FLT3 receptors as a consequence of either mutation or over-expression of wild-type *FLT3*.[21] Moreover, PKC412 also appeared to be active *in vivo*, efficiently targeting human *MLL* rearranged ALL cells over-expressing wild-type *FLT3* in mice.[21] The present study was designed to explore FLT3 as a therapeutic target in primary patient samples from infants with MLL. Therefore, we measured *FLT3* as well as *FLT3L* mRNA expression levels in a large cohort of infant MLL patients and compared these to the expression levels of these genes in both infants and older children (non-infants) with ALL carrying germ line *MLL* genes. Additionally, the cytotoxic effects of PKC412 were determined in primary infant MLL and non-infant ALL samples. Finally we assessed the infant MLL samples for the presence of activating mutations in FLT3, and analyzed the level of FLT3 receptor phosphorylation in patients carrying either mutated or wild-type *FLT3*.

PATIENTS, MATERIALS & METHODS

Patient samples

Primary bone marrow and/or peripheral blood samples from untreated infants (<1 year of age) initially diagnosed with ALL were collected at the Erasmus MC - Sophia children's hospital and other hospitals participating in the INTERFANT-99 treatment study. Samples from pediatric ALL patients older than 1 year of age (non-infants) were obtained either from the German Cooperative ALL (COALL) study group or the Erasmus MC - Sophia Children's Hospital. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/ml ; Nycomed Pharma, Oslo, Norway), centrifuged at 480 g for 15 minutes at room temperature. Isolated mononuclear cells were washed twice in PBS and resuspended in RPMI 1640 medium (Dutch modification without L-glutamine ; Invitrogen life technologies, Breda The Netherlands) supplemented with 20% fetal calf serum (FCS ; Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Invitrogen) 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (ITS media supplement ; Sigma, St Louis

MO, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.125 μg/ml fungizone and 0.2 mg/ml gentamycin (Invitrogen). Contaminating non-leukemic cells were removed using immunomagnetic beads as described by Kaspers *et al.*[29] All samples used contained >90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins.

Patient characteristics were collected in reference labs of the INTERFANT-99 and the COALL study groups. As part of the INTERFANT-99 study, the infant ALL samples were screened for the presence of MLL rearrangements by FISH analysis, and the type of translocation determined using PCR. In the present study 35% of the infant ALL samples carried a t(4;11), 28.3% a t(11;19), 10% a t(9;11) and 13.3% had germ line *MLL* genes. The remaining 13.3% of the samples either carried other less frequently found translocations involving the *MLL* gene, or PCR analysis could not be performed due to lack of material.

RNA and DNA extraction

Total RNA and genomic DNA were extracted from a minimum of 5 x10^6 leukemic cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions with minor modifications. Quantification of both RNA and DNA was performed using a spectrophotometer and the integrity of the extracted RNA was assessed on 1% agarose gels.

Quantitative real-time PCR (TaqMan©)

Extracted RNA was reverse transcribed and the obtained cDNA was used to quantify *FLT3* and *FLT3L* mRNA expression relative to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), using quantitative real-time PCR (TaqMan©) as described previously.[30] Primer and probe combinations used to amplify and detect *FLT3*, *FLT3L* and *GAPDH* expression are listed in Table 1.

Table 1. Primer and probe combinations used for quantitative real-time PCR (TaqMan©) analysis.

Target gene	Primer/probe	Sequence
FLT3	forward	5′-AGC ATC CCA GTC AAT CAG-3′
	reverse	5′-CTG GCT GGT GCT TAT GA-3′
	probe	5′-(FAM)-TTA AAG CCT ACC CAC AAA TCA GAT GT-(TAMRA)-3′
FLT3L	forward	5′-GAG CCC AAC AAC CTA TCT C-3′
	reverse	5′-GGA CGA AGC GAA GAC A-3′
	probe	5′-(FAM)-ATG GAG CGG CTC AAG ACT GT-(TAMRA)-3′
GAPDH	forward	5′-GTC GGA GTC AAC GGA TT-3′
	reverse	5′-AAG CTT CCC GTT CTC AG-3′
	probe	5′-(FAM)-TCA ACT ACA TGG TTT ACA TGT TCC AA-(TAMRA)-3′

Detection of *FLT3*/ITDs

Detection of internal tandem duplications (ITDs) of the JM domain of *FLT3* was performed as described by Kiyoi *et al.*[31] PCRs were carried out in a total reaction volume of 50 µl containing TaqMan buffer II (Applied Biosystems), 2 mM $MgCl_2$, 200 µM of each dNTP (Amersham Pharmacia Biotech), 300 nM forward and reverse primer,[31] 1.25 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 500 ng of genomic DNA as a 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 been shown to posses a FLT3/ITD,[32] was used a as positive control.

Detection of *FLT3* activation loop mutations

Detection of the activating mutations affecting either Asp835 or Ile836 within the activation loop of the *FLT3* gene was performed essentially as described by Yamamoto et al. (2001).[19] However, to fit our standard PCR procedure, a different set of primers (forward: 5'-TCA CCG GTA CCT CCT ACT G-3'; reverse: 5'-AAA TGC ACC ACA GTG AGT G-3') was designed to amplify the region of interest. To detect mutations, PCR products amplified as described above, were digested overnight at 37°C using the restriction enzyme EcoRV. Incomplete digested PCR products, visualized on 2% agarose gels, were extracted from the gel using the Wizard SV gel and Clean-up system (Promega, Leiden, The Netherlands). Clean undigested PCR fragments were cloned into pCR2.1 plasmids using a TA cloning kit (Invitrogen) and transformed into competent *E. coli* (DH5α) cells by heat shock. Individual clones were recovered from overnight cultures using the Wizard Plus SV Minipreps DNA purification system (Promega) and sequenced on a 310 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator v1.1 cycle sequencing protocol (Applied Biosystems), to confirm the presence of a mutation.

Detection of *FLT3* gene amplification using FISH analysis

The presence of *FLT3* amplification was determined with dual-color fluorescence in situ hybridization (FISH) analysis on cytospin preparations. Two BAC clones, 153M24 and 179F17 isolated from the human BAC library RPCI-11 (Children's Hospital Oakland Research Institute - BACPAC resources, Oakland, CA, USA), containing *FLT3* sequences were used as probes. Probes were labeled by nick translation using digoxigenin-11-dUTP for 179F17 and biotin-16dUTP for 153M24 and were hybridized and detected as previously described.[33] The hybridization mixture contained 50 ng of each labeled probe and 5 µg of human Cot-1 DNA. In all cases two independent observers examined 100 to 200 nuclei.

In vitro PKC412 cytotoxicity (MTT assay)

In vitro cytotoxicity to PKC412 was determined using the MTT assay as described previously.[34] Briefly, leukemic cells were cultured in round-bottomed 96-well microtitre plates (Greiner bio-one) in the presence of six 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-hour 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,[35] was measured at 562 nm on a spectrophotometer. Assay results were deemed successful when a minimum of 70% leukemic cells was present in the control wells after 4 days of incubation and when the control optical density exceeds 0.050.[34]

Immunoprecipitation and Western Blot analysis of FLT3 phosphorylation

Leukemic cells were cultured both in the absence and presence of 500 nM PKC412. After four hours of exposure to PKC412, cells were washed twice in ice cold PBS and resuspended in 100 μl lysis buffer composed of 25 mM Tris buffer, 150 mM NaCl, 5 mM EDTA, 10% Glycerol, 1% Triton X-100, 10 mM Sodium Pyrophosphate, 1 mM Sodium Orthovanadate, 10 mM Glycerolphosphate, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Aprotinine (Sigma), 10 mM Sodium Fluoride and 20 μl freshly prepared Sodium Pervanadate. Accordingly, cell lysis was allowed for 30 minutes on ice. Cell lysates were cleared by centrifugation for 15 minutes at 13000 rpm and 4°C. Protein concentration was determined using the BCA protein assay (Pierce Biotechnology, Inc., Rockford, USA) with different concentrations of bovine serum albumin (BSA) as standards.

For immunoprecipitation (IP), aliquots of whole cellular lysates containing 500 μg of protein were pre-cleared with 10 μl of G-plus Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and accordingly IP was performed with 10 μl of G-plus Agarose supplemented with 3 μg of rabbit polyclonal antibodies specific for the human FLT3 (Santa Cruz Biotechnology). Following denaturation in Laemmli buffer, precipitates were separated on 4-12% polyacrylamide gradient gels and separated proteins were transferred to PVDF-membranes on which tyrosine-phosphorylation was detected using 4G10 mouse antibodies (Upstate Biotechnology, Lake Placid, NY, USA) and

visualized using peroxidase-labeled secondary rabbit anti-mouse antibodies and chemiluminescence (ECL kit, Amersham, Little Chalfont, UK). To confirm FLT3 receptor expression, blots were stripped and re-probed with anti rabbit polyclonal antibodies against FLT3 (Santa Cruz).

Statistical analysis

Differences in *FLT3* and *FLT3L* expression between patient groups were statistically evaluated using the Mann-Whitney *U* test. Differences in mean cytotoxicity responses between patient groups were statistically analyzed using the Student t-test. All analyses were two-tailed, and differences were considered statistically significant at $P<0.05$.

RESULTS

Using quantitative real-time PCR we confirmed our earlier gene expression profiling data[12] : elevated *FLT3* mRNA expression levels were detected in *MLL* gene rearranged infant ALL (infant MLL) as compared to both infants and older children (non-infants) carrying germ line *MLL* genes (Figure 1a). Infant MLL patients (n=41) expressed significantly ($p<0.001$) ~16-fold higher levels of *FLT3* mRNA as compared to infant ALL patients (n=8) and ~2-fold higher than non-infant ALL patients (n=22) ($p=0.001$). No differences in *FLT3L* expression were observed between infant MLL (n=33), infant ALL (n=4) and non-infant ALL (n=10) patients (Figure 1b).

PKC412 has been shown to be cytotoxic to lymphoblastic leukemia cell lines carrying *MLL* gene rearrangements. In the present study we investigated the effects of PKC412 on primary infant MLL cells. *In vitro* PKC412 cytotoxicity was determined in 29 infant MLL and 19 non-infant ALL patients, as well as in five AML patients carrying FLT3/ITDs. Figure 2a shows the mean cytotoxic response at four increasing concentrations of PKC412. At PKC412 concentrations ranging from 40 nM to 375 nM, no response is observed in the non-infant ALL samples, whereas these concentrations of PKC412 increasingly induced leukemic cell death in the infant MLL samples (Figure 2a). The differences in cytotoxic response to PKC412 between infant MLL and non-infant ALL samples were statistically significant at PKC412 concentrations of 125 nM and higher ($p\leq0.01$). Moreover, the cytotoxic response to PKC412 in infant MLL was comparable to the response observed in FLT3/ITD positive AML samples (Figure 2a). Using the median *FLT3* expression from Figure 1 as the cut-off value, the infant MLL samples for which both *FLT3* expression and PKC412 cytotoxicity data was obtained were divided into two groups expressing either low or high levels of *FLT3* transcripts. As shown in Figure 2B, the cytotoxic response to PKC412 is more pronounced in infant

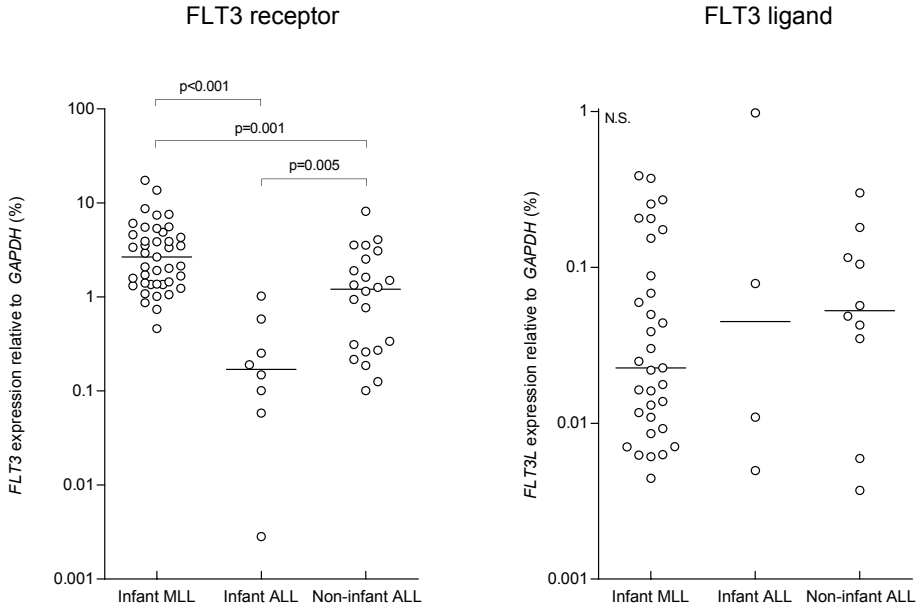

Figure 1. Relative *FLT3* and *FLT3L* expression in childhood ALL with and without *MLL* gene rearrangements

A. Relative mRNA expression of the Fms-like tyrosine kinase 3 (FLT3) as determined by quantitative real-time PCR (TaqMan) in infants with MLL (n=41) and both infants (n=8) and non-infants (n=22) with ALL harboring germ line *MLL* genes. **B.** Relative FLT3 ligand (*FLT3L*) expression in primary infant MLL (n= 33), infant ALL (n=4) and non-infant ALL (n=10) samples. Open circles (O) indicate individual patients and lines (—) median expression values. Differences in *FLT3* and *FLT3L* expression between two patient groups were statistically analyzed using the Mann-Whitney U test. N.S. means that the p-value was higher than the 0.05 in all comparisons.

MLL samples expressing high *FLT3* mRNA levels as compared to infant MLL samples expressing lower levels of *FLT3*, although these differences did not reach statistical significance.

To investigate whether activating mutations in *FLT3* could explain the observed sensitivity to PKC412, we next assessed the infant MLL samples for the presence of both internal tandem duplications (ITDs) within the JM domain and activation loop mutations affecting either Asp835 or Ile836. None of the 39 infant MLL samples tested appeared to exhibit a FLT3/ITD. Figure 3a shows the amplified region surrounding the FLT3/ITD in MV4;11 cells as a positive control and four examples of FLT3/ITD negative infant MLL patients. Out of 36 infant MLL samples tested only one patient (~3%) turned out to harbor a mutation within the activation loop FLT3. Figure 3b shows incomplete *EcoRV* digestion of the amplified region harboring codons Asp835 and Ile836 (encompassing the *EcoRV* recognition sequence) within the FLT3 activation loop from this patient. Complete digestion of this PCR product from a patient sample negative for this type of mutation is also shown. Sequence analysis of the mutation

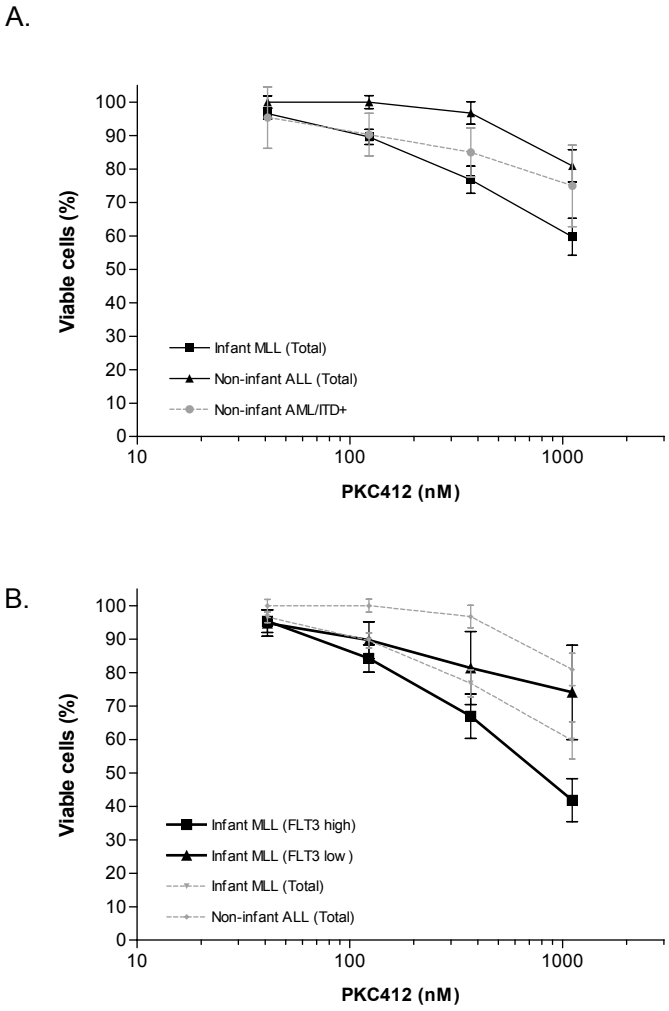

Figure 2. *In vitro* PKC412 cytotoxicity in childhood ALL with and without *MLL* gene rearrangements
A. MTT dose-response curves showing the mean cytotoxic response to PKC412 in primary leukemic cells from infants with MLL (n=29), non-infants with ALL (n=19) and FLT3/ITD positive AML patients (n=5). **B**. MTT dose-response curves showing the mean cytotoxic response to PKC412 in infant MLL samples expressing high levels of FLT3 (n=9) compared to infant MLL samples expressing lower FLT3 levels (n=8). Error bars represent standard error of the mean (SEM).

revealed that this patient carried a three-nucleotide (CAT) deletion that results in a deletion of isoleucine 836 (Δ836) (Figure 3c).

Since the frequency of known activating *FLT3* mutations in our cohort of infant MLL patients appeared to be very low, whereas the infant MLL cells are more sensitive to the FLT3 inhibitor PKC412 as compared to leukemic cells from non-infant ALL patients (Figure 2), we asked whether high-level expression of *FLT3* is sufficient to autophosphorylate and thus activate FLT3 in the absence of activating mutations. To study

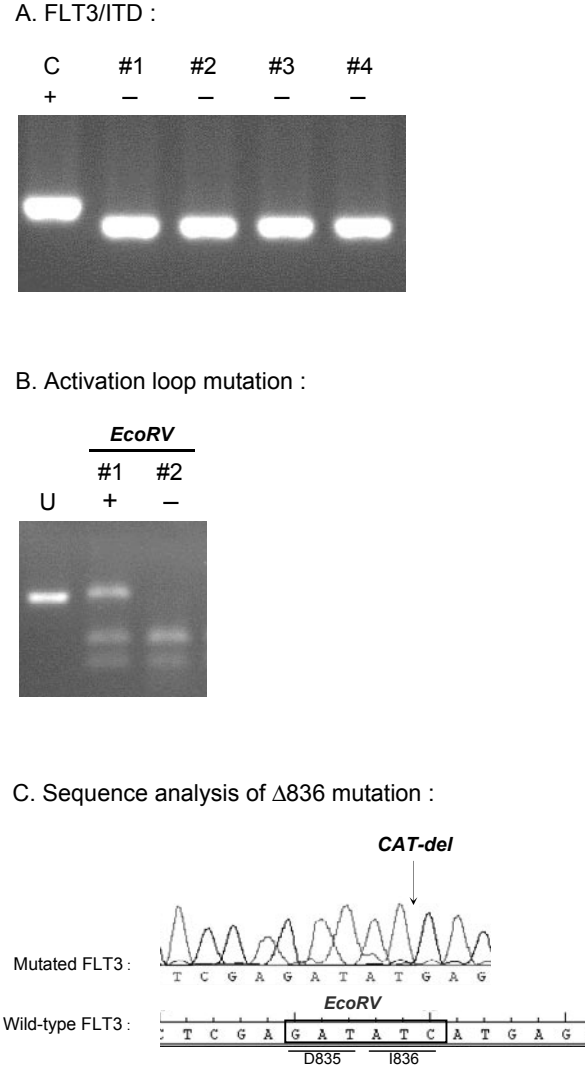

Figure 3. FLT3 activating mutations in infant MLL
A. Internal tandem duplication (ITD) of the juxtamembrane domain of FLT3 in the MV4-11 cell line (C; positive control) and four examples of infant MLL patients negative for FLT3/ITDs as determined by PCR. **B.** Amplified PCR-product covering the *EcoRV* site within the activation loop of FLT3, undigested (U) and digested in both a patient positive (+) and negative (–) for this type of mutation. **C.** Sequence analysis of the activation loop mutation found in one out of 36 patients tested.

this, FLT3 phosphorylation was assessed in several infant MLL patients displaying varying levels of *FLT3* expression as well as in a non-infant ALL patient (Figure 4). The first three patients express high levels of wild-type *FLT3* (as determined by sequence analysis of the entire *FLT3* gene) and exhibit significant levels of FLT3 posphorylation and thus activation, which was completely reversed upon exposure of the leukemic cells

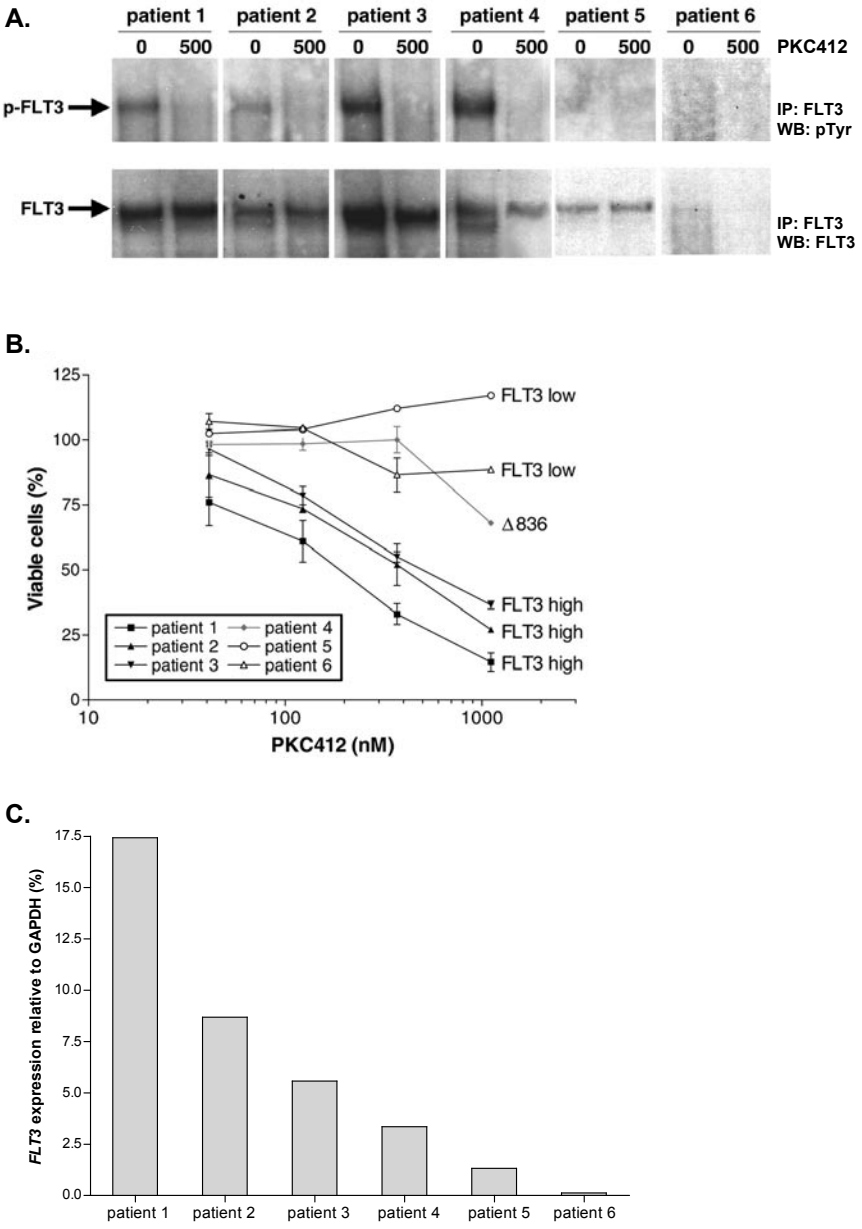

Figure 4. Relation between high-level *FLT3* expression, FLT3 activation and *in vitro* sensitivity to PKC412

A. Immunoprecipitation analysis of FLT3 in primary infant MLL cells carrying wild-type (patients #1-3 and #5) or mutated (patient #4) FLT3, cultured for 4 hours in the absence and presence of 500 nM of PCK412. Patient #6 represents a non-infant ALL patient. To determine the phosphotyrosine content of FLT3, immunoblots were probed with anti-phosphotyrosine (4G10) and with anti-FLT3 to assess FLT3 loading. **B.** MTT dose-response curves showing the mean cytotoxic response to PKC412 for the individual patients. Error bars represent standard error of the mean (SEM) of duplicate wells. **C.** Representation of the *FLT3* expression levels for the individual patients.

to 500 nM of PKC412 for four hours (Figure 4a). Patient #4 represents the one infant MLL patient in which the Δ836 activation loop mutation was identified. As expected, reversible FLT3 phosphorylation was also detected in this sample (Figure 4a). Patient #5 and #6 are samples from an infant MLL and a non-infant ALL patient respectively, expressing low levels of *FLT3* (Figure 4c). No FLT3 phosphorylation could be observed in these samples (Figure 4a). Figure 4b clearly demonstrates that the patients expressing high levels of wild-type *FLT3* are sensitive to PKC412, whereas the samples from patients expressing low *FLT3* levels that lack FLT3 phosphorylation do not respond to this drug. Patient #4, carrying the Δ836 activation loop mutation seems to respond only at concentrations of PKC412 above 300 nM (Figure 4b).

In the *MLL* rearranged ALL cell line SEMK2-M1, over-expression of *FLT3* has been shown to be due to amplification of the *FLT3* gene on chromosome 13q12.[21] To investigate whether gene amplifications of *FLT3* also explained high-level *FLT3* expression as observed in primary *MLL* rearranged ALL cells, we screened infant MLL (n=39) samples for *FLT3* amplifications using FISH analysis. However, none of the 39 samples tested showed amplified *FLT3*.

DISCUSSION

The class III receptor tyrosine kinase Fms-like tyrosine kinase 3 (*FLT3*) is one of the most frequently mutated genes in hematological malignancies, including both AML and ALL.[14] Internal tandem duplications (ITDs) of the juxtamembrane (JM) domain and mutations within the activation loop of the receptor appeared to constitutively activate the receptor in a ligand-independent manner, consequently promoting proliferation and survival.[19,31] It was recently shown by microarray analysis that *FLT3* is over-expressed in patients with *MLL* gene rearranged ALL (designated MLL), when compared to patients with both conventional ALL and AML lacking chromosomal abnormalities involving the *MLL* gene.[12] Furthermore, human leukemia cell lines carrying chromosomal translocations involving *MLL* and activated FLT3 are sensitive to FLT3 inhibition *in vitro* and in a mouse model of MLL.[21] In the present study we show that over-expression of wild-type *FLT3* is associated with sensitivity to PKC412 in primary infant MLL cells.

Using quantitative real-time PCR analysis we confirmed high-level *FLT3* expression in leukemic cells from patients carrying *MLL* gene rearrangements as previously shown by gene expression profiling.[12] Infant MLL patients significantly expressed higher levels of *FLT3* mRNA as compared to both infant ALL and non-infant ALL samples harboring germ line *MLL* genes. Screening the cohort of infant MLL patients for the presence of described activating mutations in *FLT3*, however, showed these mutations to be

extremely rare in this group of patients. Nevertheless, comparing the mean cytotoxic response of the FLT3 inhibitor PKC412 in leukemic cells from infant MLL and non-infant ALL patients, we observed that infant MLL patients are markedly more sensitive to this drug. To evaluate the relative sensitivity of infant MLL samples to PKC412 in respect to patient samples harboring activating mutations, we also assessed the cytotoxic response of PKC412 to several FLT3/ITD positive AML samples. Infant MLL patients appeared at least equally as sensitive to PKC412 as AML patients carrying FLT3/ITDs. Interestingly, infant MLL patients displaying high-level *FLT3* expression tended to be more sensitive to PKC412 as compared to infant MLL patient expressing lower levels of *FLT3*, suggesting a relation between the level of *FLT3* expression and sensitivity to PKC412. In addition we observed that high-level *FLT3* expression is associated with FLT3 phosphorylation, which is in concordance with previous data.[36] This suggests that high-level expression of wild-type *FLT3* may indeed be sufficient to activate the receptor in the absence of ligand binding, thereby sensitizing these leukemic cells to FLT3 inhibition.

The most frequent type of activating mutations found in acute myeloid leukemia (AML) are FLT3/ITDs, which occur in approximately 24% of the adult cases and in about 10-15% of the childhood cases.[14] In adult ALL, tandem duplications of the JM domain are rarely observed.[14] Recently Armstrong *et al*. described novel deletions within a 7-amino acid region of the JM domain of *FLT3* in 3 out of 25 (12%) children with hyperdiploid ALL.[37] In the present study, no FLT3/ITDs were observed in primary infant MLL samples, which is in concordance with data reported by Xu *et al*.[38] In contrast to *MLL* rearranged ALL, FLT3/ITD is rather frequently observed in AML patients carrying intragenic abnormalities within the *MLL* gene like partial tandem duplications.[39,40]

In comparison to previously reported frequencies, the incidence of activation loop mutations in this cohort of infant MLL samples (~3%) seems rather low. We have previously reported the incidence of activation loop mutations in another group of MLL samples to be approximately 17% (5/30).[21] Taketani *et al.* reported 8 out of 44 (~18%) infant MLL cases (less than one year of age) to harbor mutations within the activation loop.[41] Despite this discrepancy it can be concluded that the incidence of activating mutations in infant MLL is <20% (3-18%); therefore, activating mutations do not fully explain the relative sensitivity of primary leukemic cells from infant MLL patients to the FLT3 inhibitor PKC412. Recently Zheng *et al.* reported evidence that in primary AML samples expressing both wild-type *FLT3* and *FLT3L*, constitutively activated FLT3 can be detected as a consequence of autocrine signaling.[42] Theoretically this could sensitize these cells to FLT3 inhibition, creating the possibility that the sensitivity to PKC412 as observed in primary infant MLL samples might be explained by elevated *FLT3L* expression resulting in increased ligand-dependent receptor activation. However, we show that *FLT3L* expression does not statistically differs between primary infant MLL, infant

ALL and non-infant ALL samples, suggesting that it is unlikely that infant MLL cells are more sensitive to PKC412 than non-infant ALL cells due to elevated autocrine receptor activation. Therefore, the fact that infant MLL patients expressing high amounts of wild-type *FLT3* mRNA exhibit pronounced levels of phosphorylated FLT3 that was completely inhibited by 500 nM of PKC412 within a four hour exposure period, suggests that indeed over-expression of wild-type *FLT3* is sufficient to activate FLT3 in a ligand-independent manner.

Thus, over-expression of *FLT3* identifies groups of patients sensitive to FLT3 inhibition as is shown in the present study in infant MLL, as well as in AML patients as previously reported.[43] The reason for *FLT3* over-expression in MLL patients, however, remains unclear. One explanation might be that the immunophenotype of infant ALL cells usually is that of a very immature early B-lineage progenitor in which *FLT3* expression has been shown to be the highest.[14] Leukemic cells from infant ALL patients carrying germ line *MLL* genes most often show common or pre-B phenotypes, expressing lower levels of *FLT3*. The consistently high expression of *FLT3* specifically in MLL, may also suggest that *MLL* translocations influence the expression of this gene. In search for a mechanism by which *FLT3* expression is elevated in infant MLL, we screened 39 infant MLL samples for the presence of *FLT3* gene amplification like found in the *MLL* rearranged ALL cell line SEMK2 which expresses exceedingly high levels of wild-type *FLT3*.[21] However none of the patient samples appeared to harbor amplified *FLT3*, excluding gene amplification as the etiology for *FLT3* over-expression.

After the discovery of the high incidence of activating *FLT3* mutations in AML, several small molecule tyrosine kinase inhibitors were developed to target the constitutive FLT3 signal as potentially novel therapeutic drug. The efficacy of several of the most promising FLT3 inhibitors including PKC412,[28] CEP-701,[32] and SU5416[44] is currently being tested in phase II clinical trials in adult AML, and preliminary results are encouraging.[23,24,26,27] Interestingly, sensitivity towards these inhibitors seems to vary between the different types of activating mutations.[45] PKC412, a staurosporine derivative originally identified as a inhibitor of protein kinase C (PKC),[46] has been shown to be effective against activated FLT3 resulting from both ITD and mutations in the activation loop of the receptor.[45] Moreover, PKC412 has also been shown to display inhibiting activity against several other class III receptor tyrosine kinases like KDR, c-KIT and PDGFR.[46] In the present study we demonstrate PKC412 to exhibit anti-leukemic activity against primary infant MLL cells with activated FLT3 receptors, whereas these effects were not observed in infant MLL cells lacking activated FLT3. These observations imply that leukemic cell death induced upon exposure to PKC412 in these cells specifically seems due to inhibition of FLT3. However, PKC412 has been shown almost completely inhibit FLT3 phosphorylation at a concentration of 100 nM.[21] While the present study does show a statistically significant difference in mean PKC412

cytotoxicity between infant MLL (~10% leukemic cell death) and non-infant ALL (no response) patients at this dose level, the absolute difference is rather small. At higher dosages of PKC412 the differences are more pronounced, and substantial percentages of leukemic cell death are observed within the infant MLL patient group. Given that FLT3 activation should fully be inhibited at PKC412 concentrations of approximately 100 nM, the increasing amounts cell death as observed at higher PKLC412 concentrations may not specifically be a consequence of FLT3 inhibition, but may rather be due to non-specific inhibition of any of the multiple other kinases inhibited by PKC412. Therefore these data suggest that infant MLL samples are significantly more sensitive to multi-target kinase inhibition that includes the inhibition of FLT3. Moreover our data indicates that FLT3 inhibition alone may not be sufficient to induce substantial degrees of leukemic cell death in primary leukemia samples, including ITD positive AML samples. This latter may suggest that the greater degree of cytotoxicity that has been reported in similar primary MLL samples using the FLT3 inhibitor CEP-701[36] may be due to inhibition of additional targets by this agent at concentrations similar to those required to inhibit FLT3. Nevertheless, initial results from phase I/II clinical trials using PKC412 or CEP-701 as single agents in refractory adult AML (and MDS) patients show that both inhibitors exhibit potential clinical activity. PKC412, administered orally at a dose of 75 mg three times daily, decreased peripheral blood counts by 50% in 14/20 (70%) of the patients.[23] CEP-701, given orally at a dose of 60 mg twice daily, significantly reduced bone marrow and peripheral blood blasts in 5/14 (36%).[24] Thus, receptor tyrosine kinase inhibition using small molecule FLT3 inhibitors may be a potential therapeutic approach for innovative treatments for *MLL* gene rearranged infant ALL. Based upon these findings, a phase I/II study using FLT3 inhibitors in childhood leukemia is currently in preparation. Possible strategies are to use FLT3 inhibitors in relapsed MLL or selected *MLL* rearranged cases at very high risk of relapse, either as single agents or in combination with conventional chemotherapeutic drugs, as preclinical studies demonstrating synergistic effects of FLT3 inhibitors with cytarabine (Ara-C) and daunorubicin have been reported.[47]

In conclusion, the present study shows that *FLT3* expression is high in leukemic cells from infants diagnosed with *MLL* rearranged ALL, and that high *FLT3* expression likely is associated with auto-phosphorylation (activation) of FLT3. Infant MLL samples were more sensitive to PKC412 than non-infant ALL samples. However, the concentration of PKC412 required to induce substantial levels of leukemic cell death in these samples is higher than the concentration needed to completely inhibit FLT3 phosphorylation. Thus, PKC412 induced cell death in infant MLL samples likely is a consequence of multi-target kinase inhibition, including inhibition of FLT3. Therefore, inclusion of multi-target tyrosine kinase inhibitors in current treatment regimens may be a potential novel therapeutic strategy to improve outcome for infant MLL patients.

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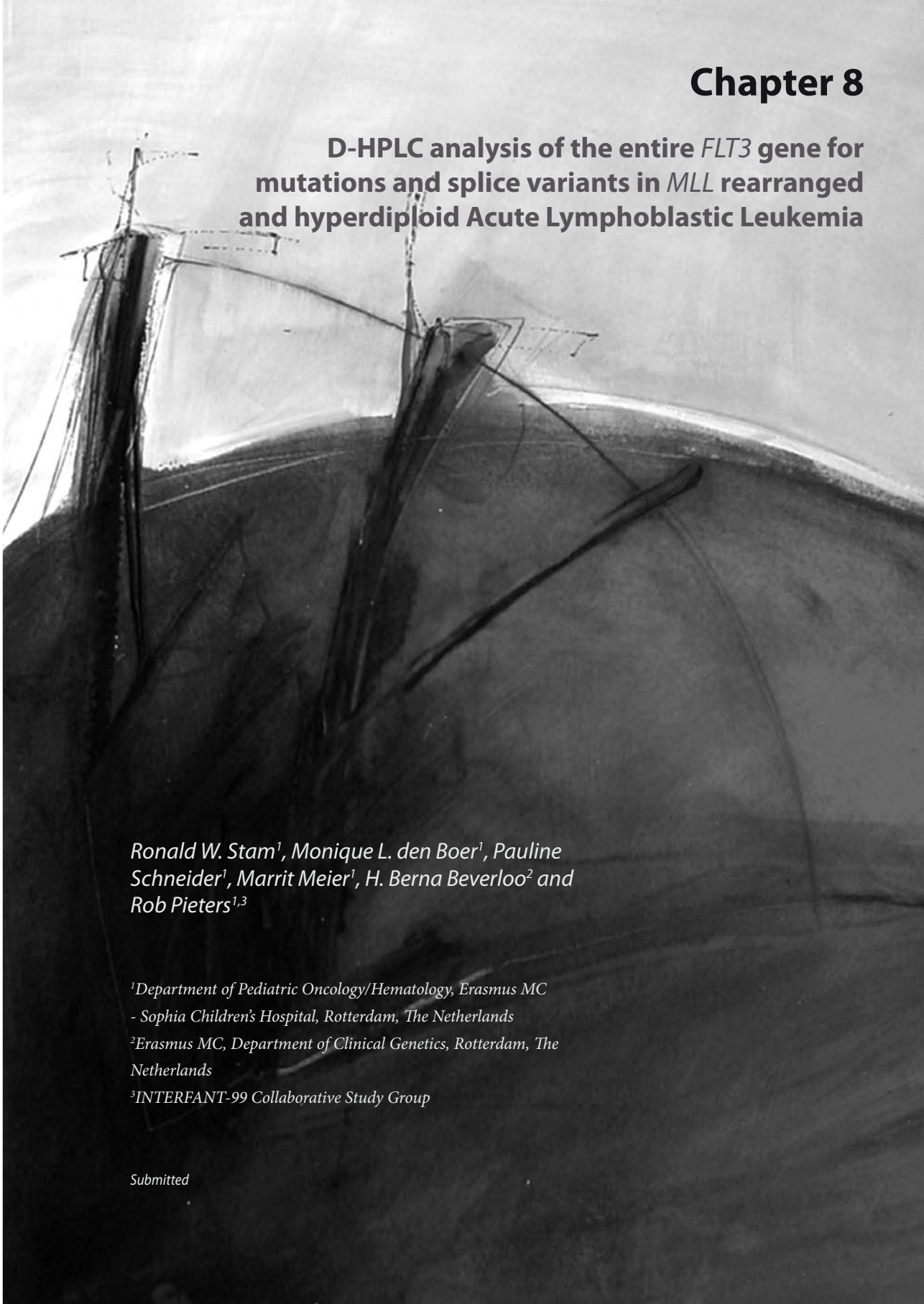
Chapter 8

D-HPLC analysis of the entire *FLT3* gene for mutations and splice variants in *MLL* rearranged and hyperdiploid Acute Lymphoblastic Leukemia



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Submitted

ABSTRACT

Both *MLL* rearranged acute lymphoblastic leukemia (MLL) and childhood hyperdiploid (HD) ALL are characterized by high-level *FLT3* expression, which has been associated with ligand-independent FLT3 activation. Lacking known activating *FLT3* mutations, these samples often are assumed to harbor wild-type *FLT3* genes. However, data on possible yet unidentified activating genetic abnormalities in other regions of *FLT3* is missing. Using D-HPLC analysis, we screened the entire *FLT3* coding sequence for the presence of mutations in infant MLL (n=45) and pediatric HD ALL (n=30) samples, as well as in mononuclear cell samples from healthy individuals (n=23). Seven small genetic abnormalities (mostly point mutations) were found in ≤10% of the infant MLL and HD ALL cases. Additionally, we observed five splice variants that (except for one) were detectable both in leukemic and non-leukemic samples. The level of expression of these isoforms (on average ~6% of total *FLT3* expression) generally was very low, suggesting that these alternative transcripts are naturally generated as by-products of normal *FLT3* transcription. Thus, evidence of prominent additional genetic alterations contributing to ligand-independent *FLT3* activation as observed in infant MLL and HD ALL is lacking, suggesting that FLT3 activation in these types of ALL mainly results from high-level expression of wild-type *FLT3*.

INTRODUCTION

As a member of the class III receptor tyrosine kinase (RTK) family, Fms-like tyrosine kinase 3 (FLT3) consists of an extracellular portion comprising five conserved immunoglobulin-like (Ig-like) domains, a transmembrane domain and an intracellular region composed of a juxtamembrane (JM) domain and a protein tyrosine kinase domain (TKD) that is interrupted by a kinase insertion domain (KID) (Figure 1). Regulating the development of hematopoietic progenitors as well as the activity of mature blood cells, class III RTKs play crucial roles in hematopoiesis.[1] FLT3 is particularly important in early B-lineage development,[2] and as such is most abundantly expressed in immature B-cells.[3]

Occurring in approximately 30%[4] of the cases, constitutively activated FLT3 as a consequence of mutation, has become a hallmark of acute myeloid leukemia (AML). Two well described types of activating *FLT3* mutations are in-frame internal tandem duplications (ITDs) within the JM domain,[5] and point mutations or small deletions/insertions either affecting codon Asp835 or Ile836 within the TKD.[6-8] Both types of mutation result in loss of the auto-inhibitory activity of the receptor,[9,10] which gives rise to ligand-independent FLT3 signaling, providing leukemic cells with a growth advantage and transforming capacity.[4,11] These findings prompted the development of small molecule tyrosine kinase inhibitors to specifically target leukemic cells that are dependent on abnormal FLT3 activity. Several of these FLT3 inhibitors (includ-

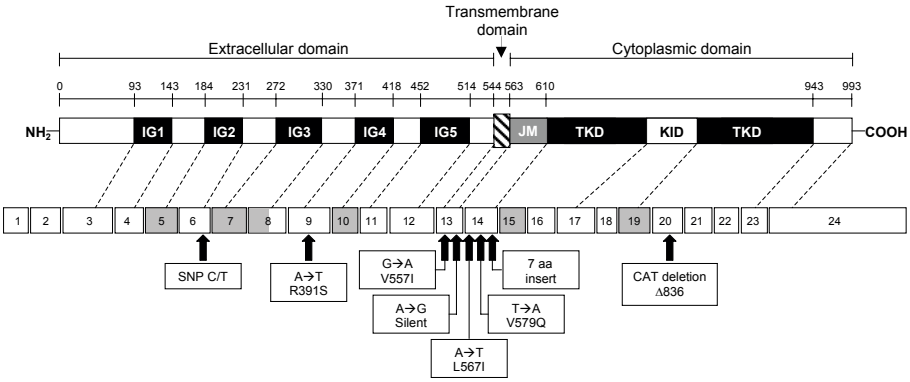

Figure 1. Schematic representation of the FLT3 receptor and its exon structure.
The FLT3 receptor consists of an extracellular domain containing five immunoglobulin-like (Ig-like) domains, a transmembrane (TM) domain and an intracellular portion consisting of the juxtamembrane (JM) domain and a protein tyrosine kinase (PTK) domain, interrupted by a kinase insertion domain (KID). The gene encoding *FLT3* comprises 24 exons. Dashed lines connect functional FLT3 domains to their corresponding exons encoding them. The position of the five Ig-like domains is based on the corresponding domains within the murine Flt3 receptor as determined by Rosnet et al[38]. Filled (gray) exons represent the deleted exons within the different splice variants, and black arrows indicate the location of each genetic abnormality.

ing PKC412, CEP-701, SU5416) have been shown to induce apoptosis *in vitro* in cells expressing different types of activating FLT3 mutations. The therapeutic potential of these inhibitors is currently being explored in phase I/II clinical trials and the initial results seem promising.[12-14]

In childhood acute lymphoblastic leukemia (ALL) the occurrence of activating *FLT3* mutations seems restricted to *MLL* gene rearranged and hyperdiploid (HD; more than 50 chromosomes) cases. In both of these subtypes of ALL, mutations within the activation loop of *FLT3* occur in 3 to 18% of the cases,[14,15] whereas FLT3/ITDs are rarely found.[16] However, in both *MLL* rearranged and hyperdiploid ALL a mechanism other than mutation appears to result in ligand-independent FLT3 activation. We and others have demonstrated that high-level *FLT3* expression, which is characteristic for *MLL* rearranged[17] and hyperdiploid ALL,[18] in the absence of ITDs and known TKD mutations, is associated with FLT3 phosphorylation (activation) and sensitivity towards FLT3 inhibitors.[19,20]

As yet, most studies mainly focused on activating ITDs and TKD mutations in *FLT3*, and leukemic samples tested negative for the presence of these mutations often are regarded as being wild-type. However, reports on novel *FLT3* mutations also resulting in ligand-independent FLT3 signaling are slowly emerging.[21,22] Therefore, the occurrence of possible unidentified activating *FLT3* mutations may challenge the proposed hypothesis that FLT3 receptor activation as observed in primary MLL and HD ALL merely is a consequence of wild-type *FLT3* overexpression.[7] Therefore, in the present study we screened the entire *FLT3* coding sequence for genetic abnormalities using Denaturing High Performance Liquid Chromatography (D-HPLC) and sequence analysis in a large cohort of primary infant MLL and childhood HD ALL samples.

MATERIALS & METHODS

Patient samples

Bone marrow and/or peripheral blood samples from untreated infants with ALL were collected at diagnosis at the Erasmus MC – Sophia Children's Hospital (Rotterdam, The Netherlands) and other hospitals participating in the INTERFANT-99 collaborative treatment protocol. Childhood HD ALL samples were collected at the Erasmus MC – Sophia Children's Hospital. Within 24 hours after sampling, mononuclear cells were isolated applying density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway). All samples contained at least 90% leukemic cells, as determined morphologically on May-Grünwald-Giemase (Merck, Darmstadt, Germany) stained cytospins. When necessary, contaminating non-malignant cells were removed using immunomagnetic beads as described elsewhere.[23] Non-leukemic mononuclear

cells were isolated from peripheral blood samples obtained from volunteering healthy adult individuals.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from a minimum of $5x10^6$ leukemic cells using TRIzol reagent (Gibco BRL, Life Technologies) according to the manufacturer's protocol. The integrity of the extracted RNA was assessed on 1% agarose gels and quantified spectrophotometrically at 260 and 280 nm. One μg of RNA was reverse transcribed into single stranded cDNA in a total reaction volume of 25 μL containing a mixture of random hexamers (2.5 μM) and oligo dT primers (20 nM), 0.2 nM of each dNTP (Amersham Pharmacia Biotech, Piscataway, NJ), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI), and 25 U RNAsin (Promega) at 37°C for 30 minutes, 42°C for 15 minutes. Finally, to inactivate enzyme activity, samples were incubated at 94°C for 5 minutes. The obtained cDNA was diluted to a final concentration of 8 ng/μL and stored at -80°C.

PCR amplification

With the exception of the first 34 nucleotides the entire *FLT3* coding sequence (NCBI accession number: U02687) was amplified as nine partially overlapping PCR products using primer combinations as listed in Table 1. All PCRs were performed in a total reaction volume of 50 μL containing 1x TaqGold buffer II (Applied Biosystems, Foster City, CA, USA), 2 mM $MgCl_2$, 200 μM of each dNTP (Amersham Pharmacia Biotech.), 300 nM forward and reverse primer, 1.25 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems) and 40 ng of cDNA (synthesized as described above) as a template. PCRs were initiated by a denaturation step at 95°C for 10 minutes, following 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Denaturing High performance Liquid Chromatography (D-HPLC) analysis

PCR products were denatured at 95°C for 5 minutes and slowly cooled down to 4°C to allow heteroduplex formation. Subsequently, 10 μL aliquots of PCR product were injected under temperature and acetonnitrile gradient conditions as listed in Table 1, and analyzed for the presence of mutations by Denaturing High performance Liquid Chromatography (D-HPLC) using the WAVE™-system 3500HT (Transgenomic Inc., Omaha, NE, USA). Abnormal PCR products were identified by examination of the WAVE patterns using Navigator software (Transgenomic). PCR products suspected to harbor genetic abnormalities were sequenced. Sequence analysis was performed on a 3100 Genetic Analyser (Applied Biosystems) using the BigDye Terminator v1.1 cycle sequencing protocol (Applied Biosystems).

Table 1. Primer combinations and D-HPLC analysis conditions.

PCR	Sequences†	First nt§	Temp (°C)#	Acetonitrile gradient (%)*
PCR 1				
Forward	5′-CGC TGC TCG TTG TTT TT-3′	92	57	56.7 – 65.7
Reverse	5′-TTG GGT TTC TGT CAT TTT CA-3′	468		
PCR 2				
Forward	5′-GCC CCA GGG AAC ATT T-3′	346	57	58.8 – 67.8
Reverse	5′-AAG GGT TCC CCT ACT TTA AGA-3′	860		
PCR 3				
Forward	5′-CAG GGG GAA AGC TGT AAA-3′	661	56	59.2 – 68.2
Reverse	5′-GAA GGT CCA CGT ACA TCT GA-3′	1212		
PCR 4				
Forward	5′-AAA GCA TCC CAG TCA ATC A-3′	1056	56	57.4 – 66.4
Reverse	5′-TGG GAG ACT TGT CTG AAC AC-3′	1474		
PCR 5				
Forward	5′-AAG CAT CGG CAA GTC AG-3′	1388	58	58.3 – 67.3
Reverse	5′-TCC CAT TTG AGA TCA TAT TCA-3′	1868		
PCR 6				
Forward	5′-ATG AAA GCC AGC TAC AGA TG-3′	1772	56	58.1 – 67.1
Reverse	5′-GGG GTA AAA ACT GAA ATT GTG-3′	2238		
PCR 7				
Forward	5′-GGG CGT GCA CAC TGT-3′	2093	57	58.1 – 67.1
Reverse	5′-AGC CAA TCC AAA GTC ACA TA-3′	2555		
PCR 8				
Forward	5′-AAG TCG TGT GTT CAC AGA GAC-3′	2470	58	56.8 – 65.8
Reverse	5′-GGC CGT TTC CTT GAG TC-3′	2858		
PCR 9				
Forward	5′-TTC CGG TTG ATG CTA ACT T-3′	2732	57	57.5 – 66.5
Reverse	5′-TGA AGC AGC AGT TGA TAA TAG AT-3′	3155		

†All oligonucleotides were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CA). §The position of the first nucleotide (nt) given for each primer is based on the complete coding sequence for *FLT3* with NCBI accession number U02687. #Temperatures used for D-HPLC analysis for indicated PCRs. *% WAVE Optimised TEAA buffer B (Transgenomic) from time 0.5 – 5 minutes.

Quantitative real-time PCR

Quantitative real-time PCR (TaqMan©) analysis was performed on an ABI Prism 7700 sequence detection system (Applied Biosystems). Using primer combinations as listed in Table 2, specific amplification of either the wild-type or the alternatively spliced *FLT3* transcript (lacking exon 7 and exon 8 partially) as well as of the housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was achieved and detected using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland).

PCRs were carried out in total reaction volumes of 50 μL containing 25 μL SYBR Green Master mix (Finnzymes) supplemented with 3 μL $MgCl_2$ (25 mM), 300 nM forward and reverse primer, 1x ROX reference dye (Finnzymes) and 40 ng cDNA (synthesized as described above) as template. Relative mRNA expression of both the wild-type and alternatively spliced *FLT3* transcripts was calculated using the comparative cycle time (C_t) method.[24] Briefly, target PCR C_t values, i.e. the PCR cycle number at which emitted fluorescence exceeds 10x the standard deviation (SD) of base-line emission, was normalized to the *GAPDH* PCR C_t value by subtracting the *GAPDH* C_t value from the target PCR C_t value, which yields the ΔC_t value. From this ΔC_t value, mRNA expression relative to GAPDH for each target PCR was calculated using the following equation :

$$\text{Relative mRNA expression (\%)} = 2^{-\Delta Ct} \times 100\%.$$

Table 2. Primer sequences used for quantitative real-time PCR (TaqMan©) analysis.

Target gene	Sequence
FLT3	
Wild-type transcript	
Forward	5′-GGC TGT TCA CAA TAG ATC TAA A-3′
Reverse	5′-AGA ATC CGT ATC ATA GTT CTG TT-3′
FLT3	
Alternatively spliced transcript	
Forward	5′-GCC CTG GTC TGC ATA TC-3′
Reverse	5′-CTT GCC ACT ATT GTG AAC AG-3′
GAPDH	
Forward	5′-GTC GGA GTC AAC GGA TT-3′
Reverse	5′-AAG CTT CCC GTT CTC AG-3′

RESULTS

Identification of (novel) *FLT3* mutations and the detection of a described SNP

Using nine partially overlapping PCRs we amplified the coding sequence of the *FLT3* gene in leukemic cells obtained from 45 infant MLL and 30 childhood HD ALL patients, as well as in mononuclear cell samples from 23 healthy adult individuals. All PCR products were screened for the presence of mutations using Denaturing High Performance Liquid Chromatography (D-HPLC). A total of eight different abnormal D-HPLC patterns were observed, all of which corresponded to genetic alterations as determined by sequence analysis. Seven of these abnormal D-HPLC patterns corresponded to actual mutations, including five different point mutations, a known activating TKD mutation, and a seven amino acid insert in the vicinity of described FLT3/ITDs (Tables 3 and 4). The remaining abnormal D-HPLC pattern appeared to correspond to a described

single nucleotide polymorphism (SNP) (NCBI: rs1933437). With the exception of this SNP, none of these genetic abnormalities were observed in mononuclear cell samples from healthy individuals.

One of the five observed point-mutations, i.e. the A→G at codon L561 which resides within the transmembrane (TM) domain, was a synonymous mutation which had been described before.[25] This silent mutation was found in 3 of 45 (7%) infant MLL and 2 of 30 (7%) HD ALL samples. The remaining four point-mutations all resulted in amino acid substitutions. Within the infant MLL patient group (n=45) one patient (2%) carried an A→T mutation that resulted in the substitution of the basic amino acid arginine (R) for a polar (uncharged) serine (S) at codon 391 within the fourth Ig-like domain (Table 3). Another infant MLL patient (2%) had a G→A mutation, replacing valine (V) for isoleucine (I), both of which are non-polar, hydrophobic amino acids, at codon 557 within the TM domain (Table 3). Within the childhood HD ALL patient group (n=30), two point-mutations were found, both located within the JM domain of FLT3. One patient (3%) carried an A→T mutation resulting in the substitution of basic amino acid lysine (K) for a non-polar (hydrophobic) isoleucine (I) at codon 567 (Table 4). In another HD ALL sample (3%) a T→A mutation was detected changing the non-polar (hydrophobic) amino acid valine (V) into a polar (uncharged) glutamine (Q) at codon 579 (Table 4).

Table 3. Novel and described *FLT3* mutations identified in primary infant MLL samples.

Mutation	Frequency[§]	Exon	Amino acid change	Location	Described before
A→T	2% (1/45)	9	R391S	Ig-like domain 4	No
G→A	2% (1/45)	13	V557I	TM domain	No
A→G	7% (3/45)	13	Synonymous (L561)	TM domain	Yes, in AML[25]
CAT del	2% (1/45)	20	Δ836	JM domain	Yes, in MLL[7]

[§]6/45 (~13%) infant MLL patients carried a mutation. In 3/45 (~7%) cases the mutation resulted in an amino acid change.

Table 4. Novel and described *FLT3* mutations identified in primary childhood HD ALL samples.

Mutation	Frequency[§]	Exon	Amino acid change	Location	Described before
A→G	7% (2/30)	13	Synonymous (L561)	TM domain	Yes, in AML[25]
A→T	3% (1/30)	14	K567I	JM domain	No
T→A	3% (1/30)	14	V579Q	JM domain	No
Insertion	3% (1/30)	14	7 aa insert at D586	JM domain	No

[§]5/30 (~17%) childhood HD ALL patients carried a mutation. In 3/30 (~10%) cases the mutation resulted in an amino acid change.

One infant MLL patient (2%) was found to harbor the known *FLT3* activating Δ836 mutation, which is a three base pair (CAT) deletion affecting codon I836 within the activation loop of the TKD.[7] In one childhood HD ALL sample (3%) a seven amino acid insertion was identified at codon 586 within the JM domain, which is closely near the insertion site at which most FLT3/ ITDs have been identified (Figure 2). Thus, leukemia

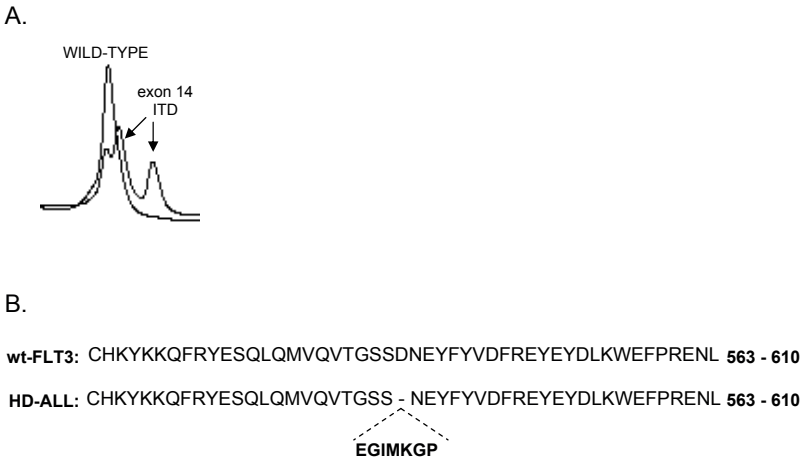

Figure 2. Internal tandem duplication found in a childhood HD ALL patient.
A. D-HPLC patterns characteristic for the internal tandem duplication (ITD) within the FLT3 juxtamembrane domain, as observed in a HD ALL sample compared to the wild-type pattern of the same region. B. Sequence analysis of the FLT3 juxtamembrane (JM) domain, showing a seven amino acid (21 bp) insert in this patient.

specific mutations leading to amino acid changes occurred in 3/45 (~7%) of the infant MLL and 3/30 (10%) of the HD ALL cases.

The D-HPLC pattern associated with the SNP (alleles C/T) in exon 6 identified heterozygous patients only. Based on the D-HPLC patterns, no discernment could be made between patients homozygous for either the C or the T allele. The average estimated heterozygosity for this SNP is 0.487 (NCBI: rs1933437). The frequency of heterozygous samples within the infant MLL and the childhood HD ALL patient groups were 44% and 33% respectively. Among the samples from the healthy controls, 38% appeared to be heterozygous for this SNP.

Identification of *FLT3* splice variants

In addition to the above mentioned genetic abnormalities, we found that five of the nine PCRs covering the *FTL3* gene, generated additional transcripts that were substantially shorter than the wild-type products (Figure 3a en 3b). Sequence analysis showed that these shorter products correspond to specific *FLT3* transcripts lacking entire exons (Table 5). Amplification of these regions on genomic DNA from corresponding patients only detected undeleted (wild-type) sequences, suggesting that these truncated transcripts are splice variants.

Interestingly, with the exception of the deletion of exon 15, which was observed in only one out of 45 (~2%) infant MLL samples, the other four splice variants were present in all samples (leukemic and non-leukemic) tested. Out of these five splice variants, two remained in frame i.e. the deletion of exon 15 affecting the tyrosine kinase domain,

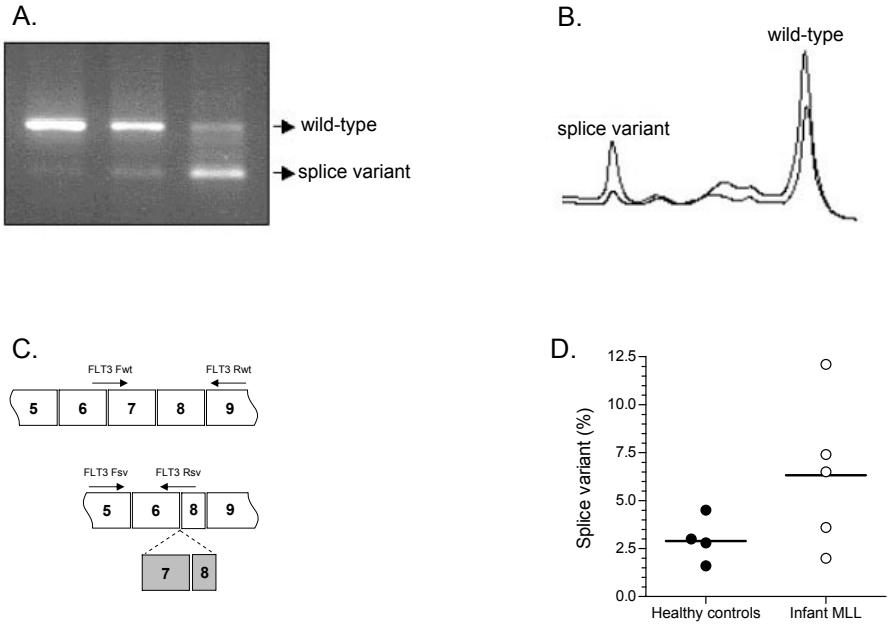

Figure 3. Detection and quantification of one of the FLT3 splice variants

Detection of the FLT3 splice variant devoid of exon 7 and part of exon 8 by A. agarose gel electrophoresis and B. D-HPLC analysis. C. Primer combinations for quantitative real-time PCR specifically amplifying either the wild-type (FLT3 Fwt and FLT3 Rwt) or the alternatively spliced transcript (FLT3 Fsv and FLT3 Rsv). D. Quantification of the splice variant was performed by applying quantitative real-time PCR analysis on four mononuclear cell samples from healthy individuals and leukemic cell samples from five infant MLL patients that ostensibly expressed the highest levels of this isoform.

and the deletion of exon 7 plus part of exon 8 affecting the third Ig-like domain (Figure 1 + Table 5). The other three splice variants were not in-frame and thus are likely to generate truncated FLT3 receptors.

The intensity of PCR products on agarose gels as well as the height of D-HPLC patterns corresponding to these PCR products are poor estimates for the actual quantity of template sequences originally present within each sample. Therefore, using primer combinations specifically amplifying either the deleted or wild-type form (Figure 3c), we performed quantitative real-time PCR analysis on one of the splice variants that seemed to be present most abundantly in several leukemic samples, i.e. the isoform devoid of exon 7 and exon 8 partially. This demonstrated that the percentage of splice variant present as part of the total amount of PCR product (both deleted and wild-type) ranged from ~2% to 4.5% (average ~3%) in non-malignant samples, and in leukemic samples ranged from ~2% to 12% (average ~6%) (Figure 3d).

Table 5. Characterization of splice variants of *FLT3* found in infant MLL and childhood HD ALL.

Splice variant	Location	3′ Splice acceptor	Deleted exon (5′… …3′)	5′ Splice donor	Deletion length (bp)	In frame	Incidence
Deletion of exon 5	Ig-like domain 2	…AGT ATA AGA **A** S I R	at acc ctg… …ggg gaa ag N T L G E S	**CT** GTA AAG AAG… **T** V K K	130	No	100%
Deletion of exon 7 and 8 partially	Ig-like domain 3	…TTC ACA ATA **G** F T I	at cta aat… …tca tca g D L N S S	**TG** GCA AGA AAC… **V** A R N	216	Yes	100%
Deletion of exon 10	Ig-like domain 4	…AAC GGA TAC **AG** N G Y	c ata tcc… …ata aga a S I S I R	**G** GAA ACC TCA… **R** E T S	104	No	100%
Deletion of exon 15	TDK	…TTA GAG TTT **G** L E F	gg aag gta… …ctg aaa g G K V L K	**AA** AAA GCA GAC… **E** K A D	105	Yes	~2% (1/45) of infant MLL
Deletion of exon 19	KID	…CAC TCT GAA **G** H S E	at gaa att… …ttt aag tcg D E I F K S	**TG** TGT TCA CAG… **V** C S Q	128	No	100%

Nucleotide sequences in capitals are the preserved sequences, the deleted sequences are depicted in lower cases. The amino acid sequence are displayed underneath the corresponding nucleotide sequence. Bold amino acid represent altered amino acids as a result of the deletion.

DISCUSSION

Once identified as being one of the genes most frequently mutated in acute myeloid leukemia (AML), *FLT3* attracted a lot of attention, especially when the significance of these mutations became apparent. Constitutively activating FLT3 in a ligand-independent manner, these mutations appeared to be involved in the malignant transformation of leukemic cells.[4,11] As such, activated FLT3 became a promising therapeutic target in AML for which several small molecule inhibitors have been developed. Recently, several phase I/II clinical trials showed promising first results with these inhibitors against leukemic cells from adult patients with refractory AML.[12-14] Both MLL and HD ALL are characterized by high-level expression of *FLT3*,[17,18] which is associated with activated FLT3 and sensitivity to FLT3 inhibitors.[19,20,26] However, these samples often lack known activating ITD or TKD mutations in the JM domain or tyrosine kinase activation loop respectively, suggesting that wild-type *FLT3* overexpression may be sufficient for ligand-independent receptor activation. To study whether other yet unidentified abnormalities in *FLT3* contribute to ligand-independent FLT3 signaling in ALL or whether indeed high-level expression of wild-type *FLT3* is sufficient, we screened the entire *FLT3* coding region for the presence of genetic abnormalities in a large cohort of primary infant MLL and childhood HD ALL samples.

Several genetic abnormalities other than described TDK mutations and ITDs were found, some of which may potentially be involved in ligand-independent FLT3 activation. One of the genetic abnormalities was a known C→T single nucleotide polymorphism (SNP) (NCBI: rs1933437). Another point-mutation turned out to be a silent A→G alteration which was reported earlier in 3 out of 34 (9%) primary AML samples,[25] but has not been reported in ALL before. Both in infant MLL and childhood HD ALL ~7% of the patients carried this synonymous mutation. To the best of our knowledge, the remaining point mutations have not been reported previously. The T→A (V579Q) mutation found within the JM domain of one childhood HD ALL sample, however, does resemble a recently described T→C mutation that results in an alternative alanine (A) at the same codon.[27] Both of these mutations, as well as the A→T (L567I) mutation found within the JM domain in another HD ALL sample, may lead to constitutive FLT3 activation. The JM domain is believed to play an important role in the auto-inhibitory activity of the receptor and disruption of this domain for example by ITDs releases its repressive conformation, allowing ligand-independent activation.[9] However, whether point mutations, like more severe genetic abnormalities such as FLT3/ITDs, are sufficient to significantly disrupt the JM domain remains to be examined.

The impact of the remaining novel point mutations identified in the present study seems less obvious. The G→A (V557I) mutation found in an infant MLL samples results in an amino acid change within the TM domain, but the class of amino acid at

that codon remains similar, as both valine and isloleucine are non-polar (hydrophobic) amino acids. Furthermore, the fact that this mutation resides within the TM domain, suggests that it may not affect domains that appeared critical in the regulation of the receptor. However, Akin et al. (2004) recently described a patient diagnosed with mast cell disease to carry a germ line F522C mutation within the TM region of the Kit receptor protein, a class III RTK family member of FLT3. Interestingly, introducing c-kit carrying this mutation in Cos-7 cells unexpectedly revealed this mutation to induce ligand-independent activation of Kit, which could be inhibited by imatinib mesylate.[28] Similarly, although extremely rare, an activating mutation within the TM region of the non-tyrosine kinase granulocyte colony-stimulating factor (G-CSF) receptor has been shown to occur in patients with AML.[29] Both of these examples suggest a significant role for the TM domain in receptor activation. In contrast, however, three described point mutations occurring in the TM region of the fibroblast growth factor receptor 3 (FGF3), another RTK, do not seem to influence receptor activation directly but rather induced increased sensitivity of FGF3 to its natural ligand.[30]

Finally we found a R391S mutation affecting the fourth Ig-like domain in one of the infant MLL samples. The extracellular Ig-like domains of RTKs are responsible for ligand binding, and hence a conformational change as a result of this amino acid substitution may affect binding of FLT3 ligand (FLT3L) to its receptor. In correspondence, point mutations within the Ig-like region of the fibroblast growth factor receptor 2 have been reported to affect ligand binding specificity.[31] Thus, point mutations within the extracellular region may also influence ligand binding of FLT3, provided that these mutations occur at critical amino acids. However, as will be discussed below, we more often found abnormalities within the ligand binding domain of FLT3 due to deletions of entire exons.

In addition to point mutations we also identified five splice variants of the *FLT3* gene. The splice variant that lacks exon 15, deleting part of the tyrosine kinase domain (TKD), was found in only one infant MLL sample. This in-frame deletion presumably results in a major defect in tyrosine kinase activity, either constitutively activating the receptor or keeping it permanently inactive. The four remaining splice variants found in this study were present in all samples tested, both malignant and non-malignant. Most leukemic samples displayed small percentages of alternatively spliced transcripts comparable to that of mononuclear cell samples from healthy individuals, not exceeding 5%. The maximum percentage of splice variant observed in leukemic samples was ~12%, suggesting that the influence of these alternative transcripts on FLT3 activity probably is limited.

These alternatively spliced isoforms can roughly be divided in those that are deleted in- frame and those that are not (Table 5). In-frame exon deletions will result in intact FLT3 receptors lacking the deleted sequence. Those deletions that are not in-frame

probably give rise to truncated receptors, as the amino acid sequence following the deletion most likely form nonsense messages. As such, the alternatively spliced isoform devoid of exon 19, which partially encodes the kinase insertion domain (KID), presumably results in a truncated FLT3 receptor lacking the far C-terminal portion of the tyrosine kinase domain. Subsequently, this truncated receptor presumably loses its auto-phosphorylating abilities, hence can be expected to be inactive. Moreover, the KID domain has been shown to be essential for tyrosine kinase activity of the Kit receptor as the lack of this domain abolishes STAT activation,[32] supporting that deletions affecting the KID domain are likely to result in receptor inactivation.

The other three splice variants affect different Ig-like domains within the extracellular region of FLT3 (Figure 1). Two of these, partially deleting the second and the fourth Ig-like domain respectively, are not in-frame. These isoforms probably generate transcripts that encode truncated FLT3 receptors devoid of intracellular domains. When translated these truncated receptors may be secreted as soluble proteins which, when their ligand-binding ability remained intact, may act as competitive inhibitors of ligand-dependent FLT3 signaling. For several class III RTKs, such a mechanism has been described. For example, a soluble truncated form of the vascular endothelial growth factor (VEGF) receptor (known as sFLT-1) also generated by alternative splicing[33] has been shown to be expressed both by leukemia and lymphoma cells.[34] Co-expression of VEGF, a positive regulator of angiogenesis, and its receptor has been observed in a variety of solid tumors and is believed to promote neo-vascularization and consequently tumor expansion via both paracrine and autocrine signaling. Soluble truncated forms of the VEGF receptor appeared to inhibit tumor angiogenesis and growth by actively competing for ligand with wild-type membrane-bound VEGF receptors.[35] Similarly, a truncated soluble form of the fibroblast growth factor receptor 4 (FGFR4) has been described that results from alternative splicing in human epithelial breast cancer cell lines MCF-7.[36] Again competitive inhibition with the wild-type receptor was observed, as the presence of soluble FGFR4 receptor neutralized the FGF-1 induced MAPK phosphorylation in these cells.[36] Thus theoretically, soluble truncated forms of FLT3 generated by the observed splice variants may intercept FLT3 ligand and thereby competitively inhibit ligand-dependent FLT3 activation in leukemic cells.

Finally, the last splice variant gives rise to a deletion of exon 7 and part of exon 8. Because this deletion is in-frame, translation of this transcript presumably generates an intact receptor lacking the third Ig-like domain almost entirely. Since the presence of five extracellular Ig-like domains, which harbor the specific ligand-binding site, is characteristic for class III RTKs, this may have drastic implications for ligand binding and specificity. For example for the class III RTK Fms it has been shown that the three N-terminal Ig-like domains constitute the high-affinity M-CSF binding region whereas the fourth and fifth Ig-like domains do not seem to be involved in ligand binding.[37] Thus

leukemic samples in which this specific isoform occurs next to the wild-type receptor may display decreased levels of ligand induced FLT3 activation.

To conclude, the observed splice variants of FLT3 presumably translate into receptors that either lack the ability to become activated or inhibit ligand-dependent activation. Therefore these splice variants rather strengthen the idea that FLT3 activation in FLT3 over-expressing infant MLL and HD ALL samples is ligand independent as ligand dependent activation is thwarted. However, given the low expression levels and the fact that these splice variants also generally occur in healthy mononuclear cells suggests that the presence of these alternative transcripts merely is a concomitant of normal *FLT3* transcription. In contrast, the leukemia-specific mutations giving rise to actual amino acid changes may potentially result in ligand-independent receptor activation or altered ligand-binding specificity. However, these mutations only occurred in ~7% (3/45) and ~10% (3/30) of the infant MLL and childhood HD ALL patients respectively. Taking into account that *FLT3* is consistently highly expressed[17] in the majority of infant MLL and HD ALL cases, and that high-level *FLT3* expression is associated with FLT3 phosphorylation,[20,26] these mutations do not explain FLT3 phosphorylation in the majority of these patients. Therefore we conclude that the newly described mutations may contribute to ligand-independent FLT3 activation in a minority of infant MLL and childhood HD ALL patients, but that in the majority of these ALL patients the constitutively activated FLT3 signal is a consequence solely of high-level expression of wild-type *FLT3*.

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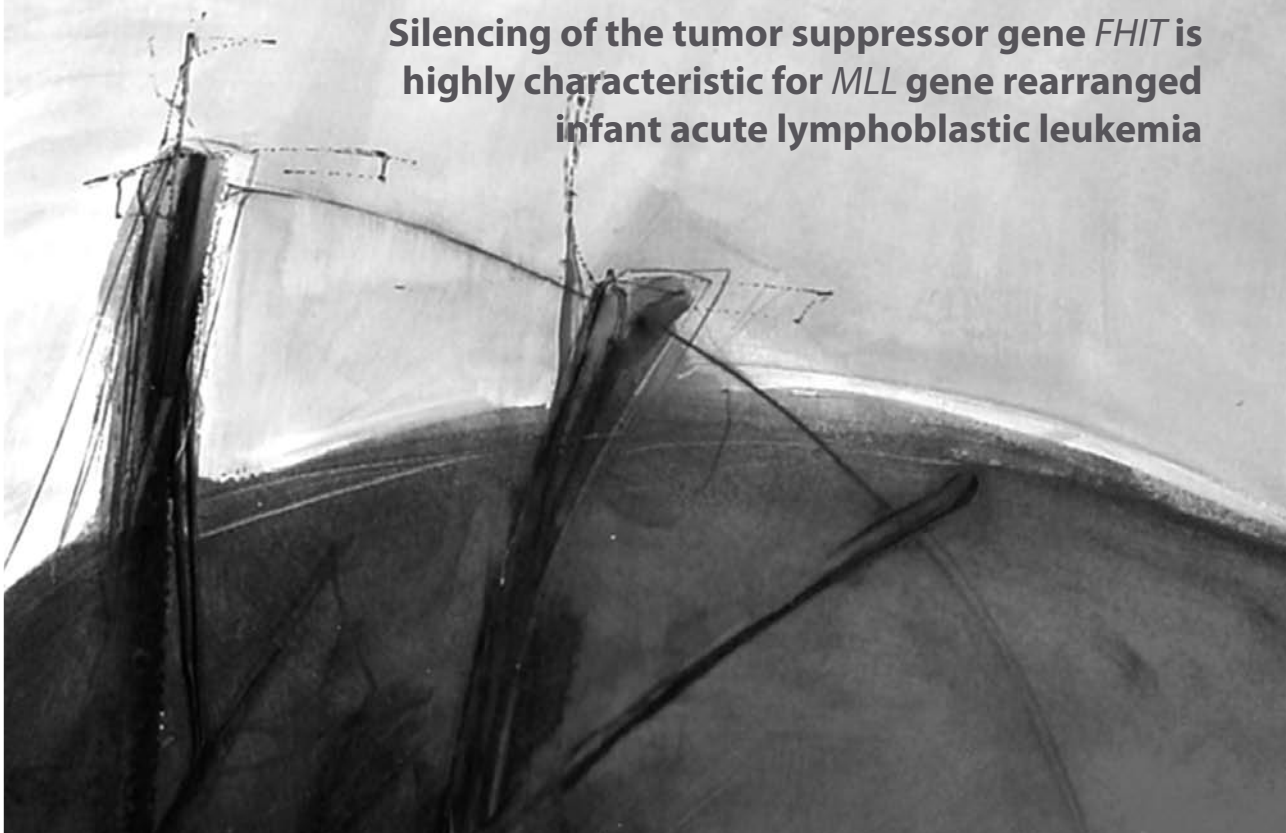
Chapter 9

Silencing of the tumor suppressor gene *FHIT* is highly characteristic for *MLL* gene rearranged infant acute lymphoblastic leukemia



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ABSTRACT

MLL rearranged acute lymphoblastic leukemia (MLL) is an aggressive type of acute lymphoblastic leukemia (ALL), diagnosed predominantly in infants (<1 years of age). Since current chemotherapy fails in >50% of patients with MLL, new therapeutic strategies are desperately needed. For this, understanding the biological features characterizing MLL is necessary. Analysis of gene expression profiles revealed that the expression of the tumor suppressor gene *FHIT* is reduced in children with *MLL* rearranged ALL as compared to ALL patients carrying germ line *MLL*. In the present study this finding was confirmed by quantitative real-time PCR. In 100% of the infant MLL cases tested, methylation of the *FHIT* 5'CpG region was observed, resulting in strongly reduced mRNA and protein expression. In contrast, *FHIT* methylation in infant and non-infant ALL patients carrying germ line *MLL* was found in only ~60% ($p \leq 0.004$). *FHIT* expression was restored upon exposing leukemic cells to the demethylating agent decitabine, which induced apoptosis. Likewise and more specifically, leukemic cell death was induced by transfecting *MLL* rearranged leukemic cells with expression vectors encoding wild-type *FHIT*, confirming tumor suppressor activity of this gene. These observations imply that suppression of *FHIT* may be required for the development of MLL, and provide new insights in leukemogenesis and therapeutic possibilities for MLL.

INTRODUCTION

In contrast to acute lymphoblastic leukemia (ALL) diagnosed in children ranging from 1 to 9 years of age, ALL in infants (<1 year of age) is associated with an exceedingly poor treatment outcome,[1,2] mainly due to cellular drug resistance.[3-5] Furthermore, ALL at infancy is characterized by a high incidence of balanced chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL, ALL-1*, or *HRX*) gene, which occur in approximately 75% of the cases.[2,4] The most commonly found translocations involving *MLL* in infant ALL include t(4;11)(p21;q23) (~60%), t(11;19)(q23;p13.3) (~20%) and, t(9;11)(p22;q23) (~5%). Infants diagnosed with *MLL* rearranged ALL have a poor prognosis (with an event free survival of ~35%),[1,2] whereas infant ALL patients carrying germ line *MLL* seem to have a far better outcome.[6,7] Recently we demonstrated that *MLL* gene rearranged ALL (designated MLL) displays a gene expression profile that is clearly distinguishable from both ALL and acute myeloid leukemia (AML) bearing germ line *MLL* genes.[8] Taken together these characteristics indicate that MLL is a distinct biological entity that responds poorly to conventional ALL-directed therapy. Therefore, in order to improve the prognosis of this disease, new therapeutic strategies are urgently needed. For this, understanding both the biology and etiology of this disease is crucial. Important aspects of the biology of MLL like high-level expression of certain *HOX* genes[8,9] and of Fms-like tyrosine kinase 3 (*FLT3*),[8-10] are now emerging and, as shown here, continue to emerge from gene expression analysis.

Gene expression profiling revealed that numerous genes are differentially expressed between ALL patients with and without *MLL* gene rearrangements, including *FHIT* of which the expression appears to be reduced in MLL (Figure 1a). The *FHIT* gene is located on chromosome 3p14.2 encompassing the most active common fragile site in the human genome i.e. the *FRA3B* locus.[11] The encoded protein FHIT is a member of the histidine triad protein (HIT) family and appears to be the human orthologue of the yeast *Scizosaccharomyces pombe* protein Aph1 as it exhibits diadenosine triphosphate hydrolase activity.[11,12] Restoring *FHIT* expression in cancer cells that lack *FHIT* expression has been shown to induce apoptosis and suppresses tumourigenicity.[13,14] Likewise, exogenous *FHIT* expression in *FHIT* negative human breast cancer cell lines enforces cells to undergo apoptosis.[15] Furthermore, treating heterozygous *Fhit* knockout mice with adenoviral constructs encoding human *FHIT*, inhibits tumor development in the fore stomach upon exposure to the carcinogen N-nitrosomethylbenzylamine.[16] Based on these and other studies, *FHIT* is hypothesized to be a tumor suppressor gene. Interestingly, *FHIT* is aberrantly expressed in a wide variety of human cancers,[17] but data on *FHIT* expression specifically in childhood acute leukemias is limited. In both cervical[18,19] and gastric cancer,[20] abnormal *FHIT* expression appeared to be a prognostic factor for an adverse outcome. However, this has not been confirmed in human adult leukemias.[21,22]

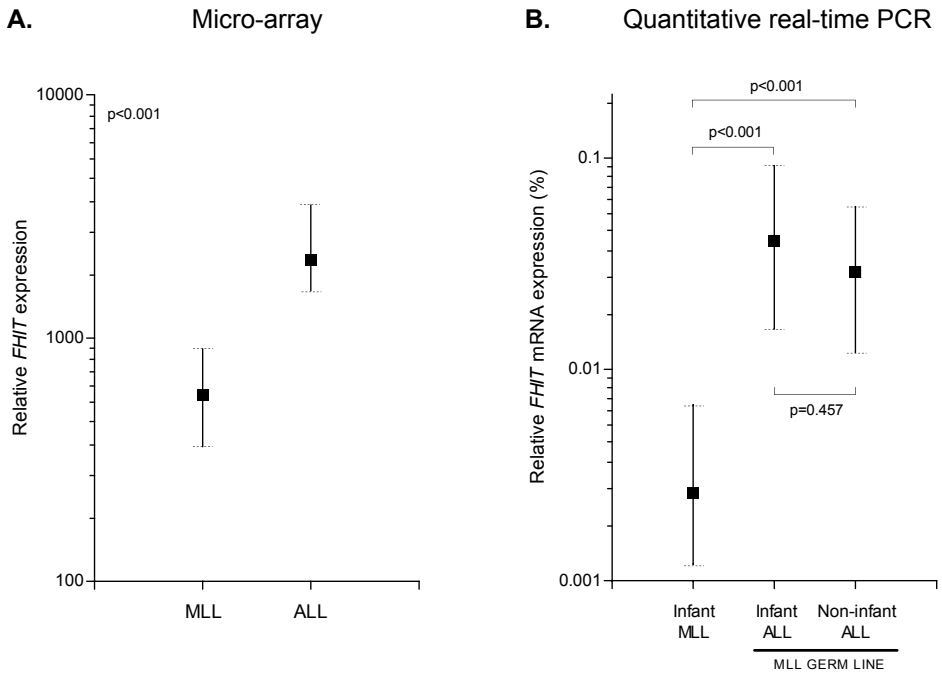

Figure 1. *FHIT* expression in childhood ALL with and without *MLL* rearrangements
Expression of the fragile histidine triad (*FHIT*) gene as determined by **A.** micro-array analysis comparing gene expression profiles from patients with MLL (n=20) and ALL (n=25) (also see Armstrong et al., (2002)), and **B.** quantitative real-time PCR relative to the housekeeping gene *GAPDH* (%) in infants with MLL (n=35) and both infants (n=8) and non-infants (n=22) with ALL harboring germ line *MLL* genes. Boxes (⊠) indicate median expression values, the dashed lines the 25th and 75th percentile respectively. Differences in *FHIT* expression between two patient groups was statistically analyzed using the Mann-Whitney U test.

In solid tumors, aberrant or absent expression of *FHIT* most often is due to loss of heterozygosity (LOH) or homozygous deletions, and less frequently to 5'CpG island methylation. Recently however, Zheng et al. (2004) demonstrated that inactivated *FHIT* as a consequence of 5'CpG island hypermethylation can be found in childhood ALL, especially in hyperdiploid and translocation-negative subtypes.[23] Here we report that in a large cohort of *MLL* rearranged infant ALL cases silencing of *FHIT* by 5'CpG island hypermethylation was observed in 100% of the cases. Furthermore we show that *FHIT* silencing could be reversed by exposing cells to the demethylating agent decitabine, which was accompanied by the induction of leukemic cell death. Finally, we demonstrate that transfecting MLL cells lacking endogenous *FHIT* expression with expression vectors encoding wild-type *FHIT* induced apoptosis and restored the tumor suppressor activity of this gene.

PATIENTS, MATERIALS & METHODS

Patient samples

Primary bone marrow and/or peripheral blood samples from untreated infants (<1 year of age) diagnosed with ALL were collected at the Erasmus MC - Sophia Children's Hospital and other hospitals participating in the INTERFANT-99 treatment protocol. Samples from ALL patients older than 1 year of age were obtained from the German COALL study. The presence of MLL gene rearrangements was assessed by RT-PCR and FISH analysis. This and other patient characteristics were collected by the involved study centers. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/ml ; Nycomed Pharma, Oslo, Norway), centrifuged at 480 g for 15 minutes at room temperature. The collected mononuclear cells were washed twice and kept in culture medium consisting of RPMI 1640 medium (Dutch modification without L-glutamine ; Invitrogen life technologies, Breda, The Netherlands), 20% fetal calf serum (FCS ; Integro, Zaandam, he Netherlands), 2 mM L-glutamine (Invitrogen) 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (ITS media supplement ; Sigma, St Louis MO, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.125 μg/ml fungizone and 0.2 mg/ml gentamycin (Invitrogen). Contaminating non-leukemic cells were removed by immunomagnetic beads as described by Kaspers et al. (1994).[24] All samples contained >90% leukemic cells, determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. For RNA and DNA isolation, a minimum of 5 x10^6 cells were lysed in TRIzol reagent (Invitrogen) and stored at -80°C until extraction.

Cell lines

All human leukemia cell lines used in this study were maintained in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) supplemented with 10% FCS (Integro), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.125 μg/ml fungizone (Invitrogen) and grown as suspension cultures at 37°C in humidified air containing 5% CO_2. Both RS4;11 and SEMK2-M1 are B lineage acute lymphoblastic leukemia cell lines carrying translocation t(4;11). MV4-11 is a myelomonocytic leukemia cell line also bearing translocation t(4;11). Jurkat, Molt-4 and CCRF-CEM all are T-ALL cell lines, and HL60 is an acute promyelocytic leukemia line. The cell lines REH and TOM-1 are TEL-AML1 and Philadelphia chromosome positive acute lymphocytic leukemias respectively and K563 is a chronic myelogenous leukemia (CML) cell line.

RNA and DNA extraction

Both total RNA and genomic DNA were extracted from a minimum of 5 x10^6 leukemic cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions,

with minor modifications.[25] The integrity of the extracted RNA was assessed on 1% agarose gels.

Quantitative real-time PCR (TaqMan©)

Extracted RNA was reverse transcribed and the obtained cDNA was used to quantify *FHIT* mRNA expression relative to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), using quantitative real-time PCR (TaqMan©) as described previously[25]. Primer and probe sequences used to amplify and detect *FHIT* and *GAPDH* expression are listed in Table 1.

Table 1. Primer and probe combinations used for quantitative real-time PCR (TaqMan©) analysis.

Target gene	Primer/probe	Sequence
FHIT	forward	5′-TGG CCA ACA TCT CAT CA-3′
	reverse	5′-ACG TGC TTC ACA GTC TGT C-3′
	probe	5′-(FAM)-TGA TGA AGT GGC CGA TTT GTT-(TAMRA)-3′
GAPDH	forward	5′-GTC GGA GTC AAC GGA TT-3′
	reverse	5′-AAG CTT CCC GTT CTC AG-3′
	probe	5′-(FAM)-TCA ACT ACA TGG TTT ACA TGT TCC AA-(TAMRA)-3′

FHIT 5′CpG methylation analysis by MSP

Exposing DNA to bisulfite converts unmethylated cytosine into uracil bases. Methylated cytosine bases, however are protected from this modification. Therefore, after bisulfite modification the nucleotide sequence of methylated DNA differs from that of unmethylated DNA. Using primers specific for the predicted sequence of bisulfite treated methylated DNA, allows the amplification of PCR products only when methylated DNA was present before bisulfite exposure. Similarly, primers specific for bisulfite treated unmethylated DNA can be designed.

For bisulfite modification, 5 μg of genomic DNA was denatured in 0.2 M NaOH for 15 minutes at 37 °C. Accordingly, 200 μl of a 100 mM hydroquinone, 5 M sodium bisulfite (Sigma) and 0.35 M NaOH (Merck) solution (pH 5) was added per μg of DNA, following a 4 hour incubation at 50°C in the dark. Bisulfite treated DNA was subsequently purified using Wizard DNA purification resin (Promega) followed by ethanol precipitation.

Methylation specific PCR (MSP) using primer sequences specific for either methylated or unmethylated *FHIT* after bisulfite treatment, was performed essentially as described by Zöchbauer-Müller et al. (2001).[26] Briefly, MSP was performed in a total reaction volume of 50 μl containing PCR buffer (Qiagen Inc., Valencia, CA, USA), 10 μl Q-solution (Qiagen), 0.4 mM of each dNTP (Amersham Pharmacia Biotech), 3 mM and 5 mM $MgCl_2$ for the methylated *FHIT* and unmethylated *FHIT* PCR respectively, 0.6 μM forward and reverse primer,[33] 1 Unit of HotStarTaq (Qiagen) and ~100 ng bi-

sulfite treated DNA as a template. Amplification was performed using a touchdown PCR with the annealing temperature decreasing from 71°C to 64°C over 14 cycles of annealing for 1 minutes and denaturation for 15 seconds at 95°C, followed by 30 cycles of 95°C for 15 sec and 64°C for 1 min. Amplified PCR products were analyzed on 3% agarose gels.

Methylation of HL60 DNA

Since the human AML cell line HL60 shows significant FHIT protein expression, bisulfite treated HL60 DNA was used as positive control for the unmethylated *FHIT* MSP. To generate a positive control for the methylated *FHIT* MSP, HL60 DNA was enzymatically methylated using the CpG methylase M.Sss I. The methylation reaction was performed in a total volume of 100 µl containing NEBuffer 2, 0.16 mM S-adenosylmethionine, 10 Units of M. Sss I methylase (New England BioLabs, Beverly, MA, USA) and 10 µg HL60 DNA, incubated at 37°C for 3 hours, followed by a 20 minute incubation at 65°C to inactivate the enzyme. Accordingly, the methylated HL60 DNA was bisulfite modified as described above.

5-aza-2′-deoxycytidine (decitabine) treatment of SEMK2 cells

To study the effects of demethylation on FHIT protein expression, SEMK2 cells were maintained in culture medium both in the absence and presence of 1 µM of the demethylating drug 5-aza-2'-deoxycytidine (decitabine) (Sigma) for 7 days. The decitabine culture medium was refreshed daily. Every 24 hours, cell viability was assessed by Annexin V staining determined by flow cytometry using a FACSCalibur (Becton Dickinson). Sampled cells were washed twice in phosphate buffered saline (PBS), and spun down cell pellets were stored at -80°C until protein extraction.

Ectopic FHIT expression in RS4;11 cells

The entire wild-type *FHIT* coding sequence was amplified using primers containing 5' extensions encoding a *Bam*HI (forward primer) or a *Bgl*II (reverse primer) restriction sites (Table 2). Accordingly, this wild-type *FHIT* transcript was cloned into the *Bam*HI/*Bgl*II site of the eukaryotic expression vector pSG5 (Stratagene, La Jolla, CA, USA).

RS4;11 cells (1 x10^7) were transfected by electroporation in 400 µL RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) containing 4 µg of either the pSG5-FHIT construct or empty vector (mock transfection), in 4 mm electroporation cuvettes (BioRad, Hercules, CA, USA). To compensate for the amount of cell death induced merely as a consequence of the electroporation procedure, control cells were electroporated in the absence of vector. Electroporation was performed using an EPI 2500 gene pulser (Fischer, Heidelberg, Germany) applying a rectangle pulse of 600 V for 2 msec. After incubating for 15 minutes at room temperature, the cells were diluted 10-fold with

RPMI 1640 (Invitrogen) supplemented with 10% FCS (Integro), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.125 μg/ml fungizone (Invitrogen) and incubated at 37°C and 5% CO_2. Cell viability was assessed by Annexin V staining determined by flow cytometry using a FACSCalibur (Becton Dickinson). Flow cytometry was also used to determine transfection efficiency of pEGFP-C1 (Invitrogen).

Table 2. Primer sequences used to amplify the entire *FHIT* coding sequence for *FHIT* cloning.

Primers	Sequence[§]
FHIT	*Bam*HI
forward	5′-*CG**G GAT CC**C GCC ACC* **ATG** TCG TTC AGA TTT GGC-3′
reverse	5′-*GA**A GAT CT**T CGG* GCG GTC TTC AAA CT-3′
	*Bgl*II

[§]Primer extensions encoding the indicated restriction sites are depicted in italic font. The actual primer sequences corresponding to the *FHIT* gene (NCBI: NM_002012) are depicted in plain font.

Protein extraction and Western Blot analysis

Cell pellets stored at -80°C were briefly thawed and resuspended in 50 μl lysis buffer composed of 50 mM Tris buffer, 150 mM NaCl, 100 mM EDTA, 1% Triton X-100, 2 mM PMSF, 3% aprotinine (Sigma), 4 μg/ml pepstatin (Sigma) and 4 μg/ml leupeptin (Sigma). Accordingly, cells were lysed for 15 minutes on ice. The supernatant of the lysed cells was cleared by centrifugation for 15 minutes at 13000 rpm and 4°C. The protein content of the cleared lysates was determined using the BCA protein assay (Pierce Biotechnology, Inc., Rockford, USA) with different concentrations of bovine serum albumin as standards.

Cell lysates containing 25 μg of protein were separated on 10% polyacrylamide gels topped with 4% stacking gels, and transferred to nitrocellulose membranes (Schleichler & Schuell, Dassel, Germany). Western blots were probed with anti-FHIT rabbit polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY, USA) or with anti-Actin mouse monoclonal antibodies (Sigma). Accordingly, the blots were labeled with either peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (DAKO, Glostrup, Denmark) and visualized using SuperSignal® West Femto chemiluminescent substrate (Pierce Biotechnology).

RESULTS

FHIT expression in childhood ALL with and without MLL gene rearrangements

More detailed analysis of our recently published micro-array study comparing gene expression profiles from *MLL* rearranged ALL (MLL) patients (n=20) with those from

patients diagnosed with conventional ALL bearing germ line *MLL* genes (n=24),[8] revealed that *FHIT* expression was significantly ($p<0.001$) reduced in MLL (Figure 1a). Using quantitative real-time PCR (Taqman) analysis we confirmed *FHIT* mRNA expression to be low in *MLL* gene rearranged infant ALL (infant MLL) as compared to both infants and older children (non-infants) not carrying chromosomal abnormalities involving the *MLL* gene (Figure 1b). Infant MLL patients (n=35) express ~15-fold less ($p<0.001$) *FHIT* mRNA compared to infant ALL patients (n=8) and 11-fold less ($p<0.001$) than non-infant ALL patients (n=22) both carrying germ line *MLL* genes.

FHIT 5'CpG island methylation analysis by MSP

Biallelic deletions in *FHIT* preferentially seem to occur in exon 5, which is the first coding exon of the *FHIT* gene.[11,27] Deletions in exon 5 usually result in reduced or aberrant expression of *FHIT*. The probe sets for *FHIT* on the Affymetrix micro-array chip HG-U95A used in the above described gene expression profiling study, do not cover exon 5 but are located in more downstream exons. In contrast, the forward primer used in the present quantitative real-time PCR analysis, is located within exon 5. Since *FHIT* mRNA could be detected using this primer, and results similar to the results from the micro-array study were obtained, we reasoned that a mechanism other that deletions in exon 5 may underlie reduced *FHIT* expression in patients with MLL.

Interestingly, all leukemia cell lines with germ line *MLL* genes tested (HL60, REH, K562, Tom-1, Jurkat, Molt-4 and CCRF-CEM) showed varying levels of FHIT protein expression (although K562 very weakly). In contrast, RS4;11, SEMK2-M1 and MV4-11, all of which harbor translocation t(4;11) involving the *MLL* gene did not express the FHIT protein (Figure 2). However, a smaller protein was detected in the RS4;11 line which may suggest the presence of truncated (aberrant) *FHIT* transcripts (Figure 2). Nevertheless, no detectable levels of *FHIT* mRNA were found in these cells. Lack of FHIT protein expression in the t(4;11) positive cell lines may be caused by 5'CpG island hypermethylation of the *FHIT* gene. To test this hypothesis we determined the methylation status of *FHIT* using a methylation specific PCR (MSP). The HL60 cell line shows pronounced levels of FHIT protein expression (Figure 2), and does not display FHIT 5'CpG methylation. As a positive control for methylated *FHIT*, enzymatically methylated HL60 DNA was used. As shown in Figure 3a, unmethylated *FHIT* is detected by in bisulfite treated HL60 DNA and weakly in enzymatically methylated HL60 DNA. Methylated *FHIT* is detected in enzymatically methylated DNA only, providing sound controls for both methlated and unmethylated *FHIT* promoters.

The frequency of *FHIT* 5'CpG island methylation in bisulfite modified DNA from a group of 87 childhood ALL patients consisting of 44 infants and 43 non-infants diagnosed with ALL (Figure 4). In 56% (24/43) of the non-infant ALL patients and 93% (41/44) of the infant ALL patients methylated *FHIT* was observed. The non-infant group

was further subdivided into a B-lineage (n=32) and a T-lineage (n=11) ALL group, of which 56% (18/32) and 55% (6/11) were positive for methylated *FHIT* respectively. The infant ALL group consisted of both of *MLL* germ line (n=8) and *MLL* rearranged (n=36) patients. The frequency of *FHIT* methylation in the 8 infant *MLL* germ line cases (63%) resembled that of the non-infant ALL cases, whereas all 36 *MLL* rearranged cases showed methylated *FHIT* ($p \leq 0.004$). Figure 3b shows *FHIT* 5'CpG methylation in three infant MLL patients bearing the three most common translocations involving *MLL*, and an infant ALL sample exhibiting germ line *MLL*, negative for *FHIT* 5'CpG island methylation.

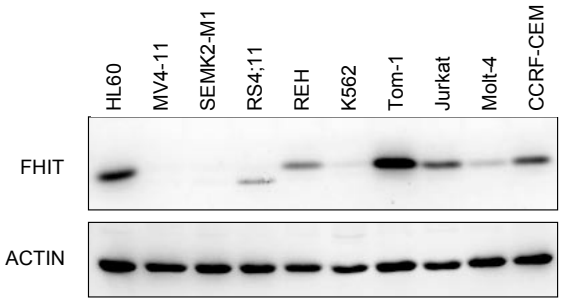

Figure 2. FHIT protein expression in human leukemia cell lines
FHIT protein expression in human leukemia cell lines as detected on Western blots probed with anti-FHIT polyclonal antibodies. The same blots were re-probed with anti-actin monoclonal antibodies to assure equal loading in each lane.

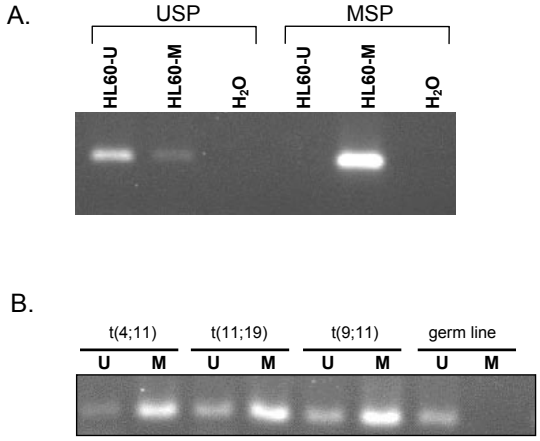

Figure 3. Methylation analysis of the *FHIT* 5'CpG region by MSP
A. Positive and negative controls for the methylation specific polymerase chain reaction. *FHIT* methylation specific PCR (MSP) and *FHIT* unmethylated specific PCR (USP) optimized on unmethylated (U) and artificially methylated (M) DNA derived from HL60 cells. **B.** *FHIT* MSP in infant ALL. Examples of *FHIT* MSP products amplified in DNA samples from infant ALL patients harboring translocation t(4;11), t(11;19), t(9;11) involving *MLL* and from a patient carrying germ line *MLL*.

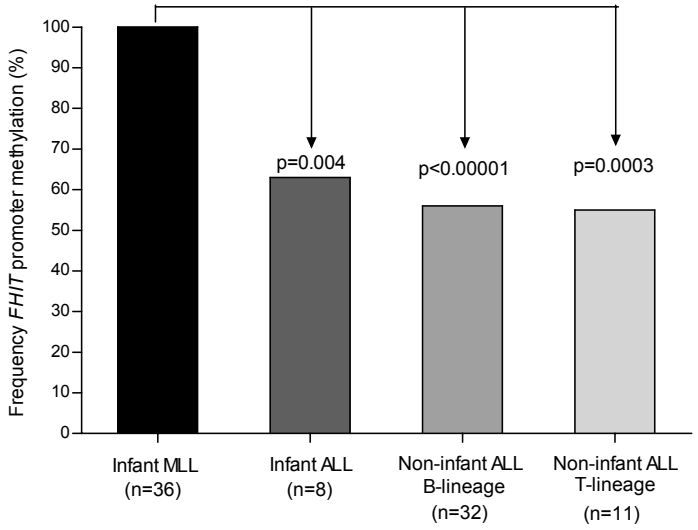

Figure 4. Frequency of *FHIT* 5'CpG hypermethylation in childhood ALL

Overview of the frequency of *FHIT* 5'CpG island methylation as determined by methylation specific PCR (MSP) in childhood acute lymphoblastic leukemia (ALL). The non-infant ALL group is further divided in to a B-lineage and T-lineage ALL group. Infants with ALL were categorized by the presence or absence of chromosomal translocations involving the *MLL* gene. Statistically significant differences in the frequency of *FHIT* methylation were observed between infant and non-infant ALL patients ($p<0.0001$) and between *MLL* rearranged and *MLL* germ line infant ALL cases ($p=0.004$) (Fisher exact test).

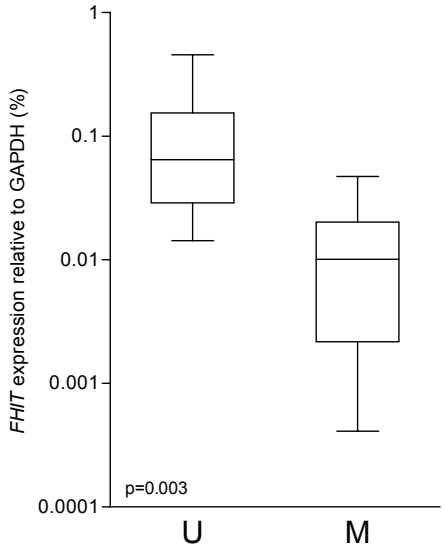

Figure 5. *FHIT* expression in methylated and unmethylated non-infant ALL

Comparison of the relative *FHIT* mRNA expression between primary non-infant ALL samples displaying either unmethylated (U) (n=8) and methylated (M) (n=8) *FHIT* 5'CpG regions. The difference in *FHIT* expression between both patient groups was statistically analyzed using the Mann-Whitney U test.

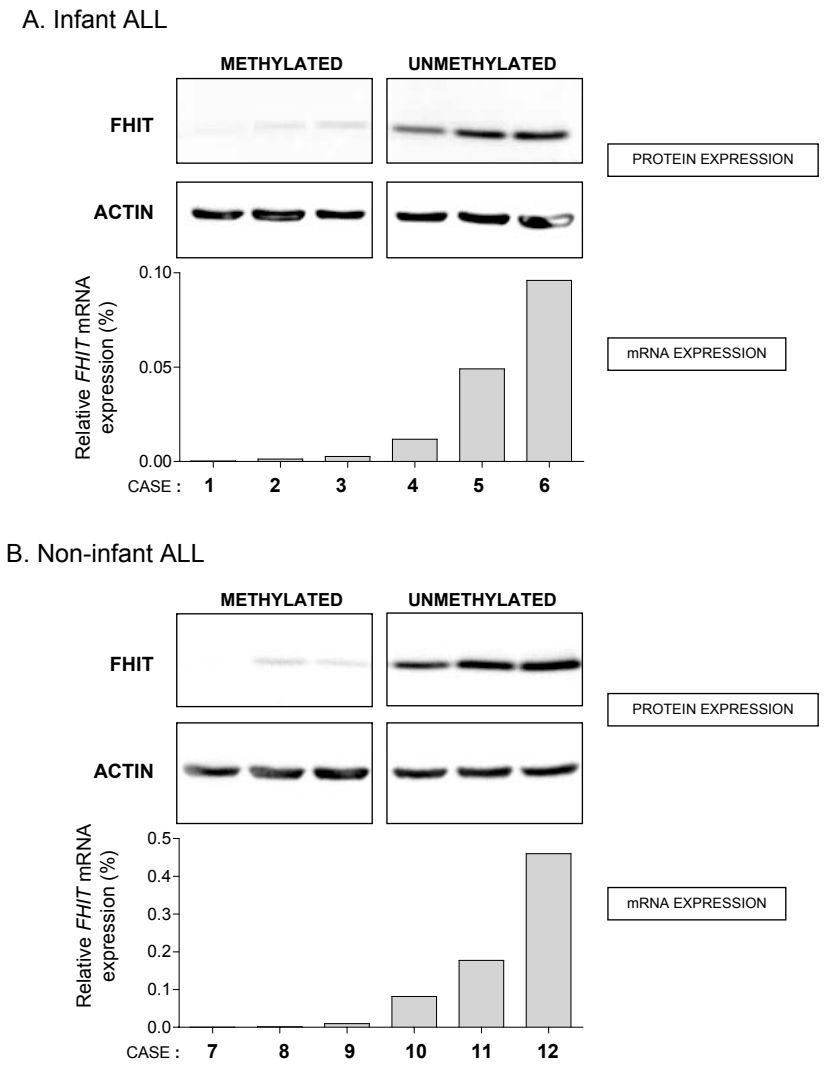

Figure 6. Relation between FHIT methylation, FHIT protein and *FHIT* mRNA expression.
FHIT protein expression assessed by Western blot analysis in samples from both **A.** infant and **B.** non-infant ALL patients exhibiting both methylated and unmethylated *FHIT* promoters (as determined by MSP). Patient samples were orderly arranged with increasing levels *FHIT* mRNA expression.

Relation between *FHIT* 5'CpG methylation, *FHIT* mRNA and FHIT protein expression

The MSP approach only allows the samples to be scored either positive or negative for the presence of methylated 5'CpG island sequences. No discrimination can be made in the degree of 5'CpG island methylation per sample. Therefore, to establish a relation between positive MSP results and reduced *FHIT* mRNA expression, we compared *FHIT* expression levels between non-infant ALL samples displaying methylated and un-

methylated *FHIT* genes, for those patients for which both expression and MSP data was available. Figure 5 shows that unmethylated samples express significantly higher levels of *FHIT* mRNA as compared to methylated non-infant ALL samples (p=0.003). Figure 6 demonstrates that methylation of 5'CpG islands within the *FHIT* gene indeed suppresses *FHIT* mRNA and FHIT protein expression in both infant and non-infant ALL patients. In infants (Figure 6a) as well as non-infants (Figure 6b) with ALL, patients with methylated 5'CpG regions only show weak expression of FHIT protein, whereas patients with unmethylated promoters express pronounced FHIT protein levels. Also, a clear relation between *FHIT* mRNA and FHIT protein expression was observed (Figure 6).

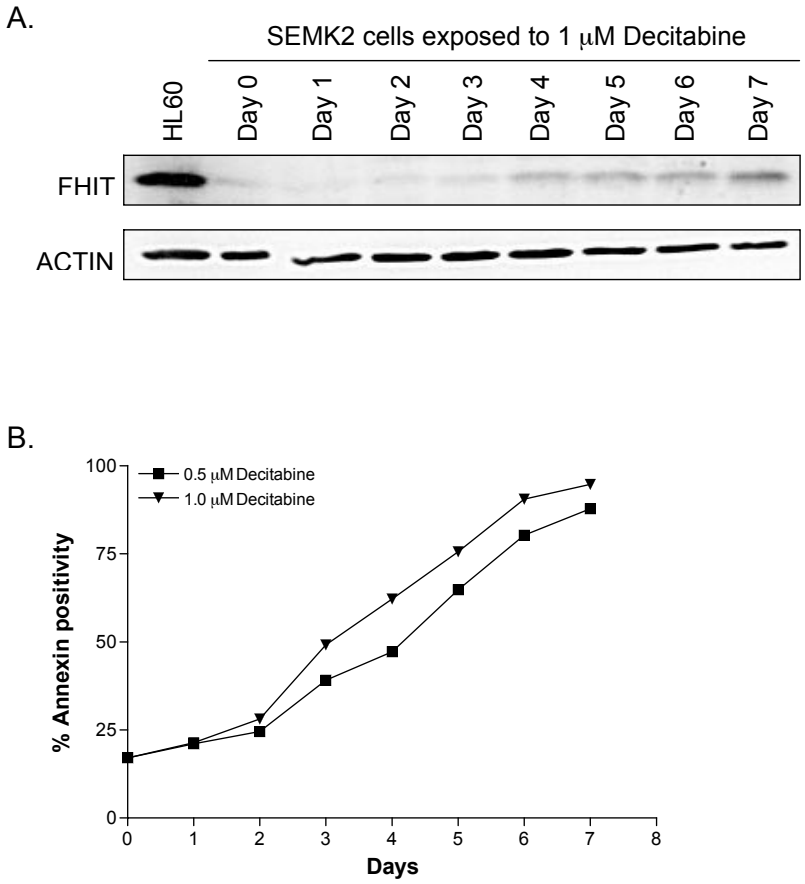

Figure 7. Re-expression of FHIT upon exposure to 5-aza-2'-deoxycytidine (decitabine).
A. Western blot analysis of FHIT protein re-expression in cells from the human leukemia cell line SEMK2-M1 continuously exposed to 10 μM of the demethylating agent 5-aza-2'-deoxycytidine (decitabine). FHIT protein expression in HL60 cells (exhibiting unmethylated *FHIT* promoters) served as a positive control. B. Apoptosis induction in SEMK2 cells upon exposure to indicated concentrations of decitabine.

Re-expression of FHIT upon exposure to 5-aza-2'-deoxycytidine (decitabine)

When *FHIT* is methylated, treatment of the cells with demethylating drugs like e.g. 5-aza-2'-deoxycytidine (decitabine), should reverse the silencing and result in the re-expression of methylated genes. Figure 7a shows that FHIT protein expression re-emerges in SEMK2-M1 cells after 6 days of exposure to 1 μM of decitabine treatment and successively becomes stronger in the following period of time. Treatment of SEMK2 cells with 1 or 0.5 μM decitabine markedly induced apoptosis (Figure 7b).

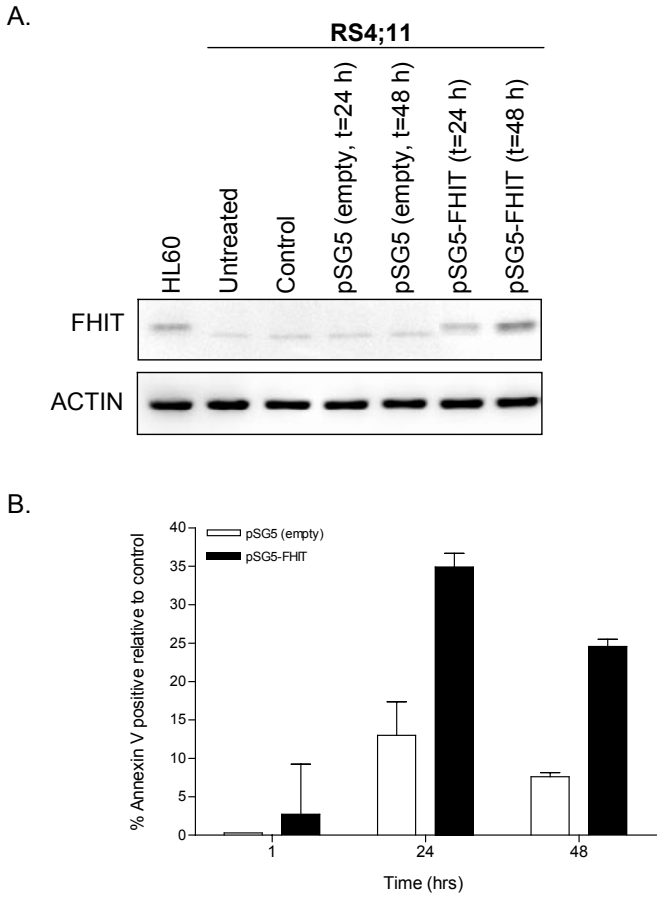

Figure 8. Effects of ectopic *FHIT* expression in RS4;11 cells.
A. Western blot analysis of FHIT protein expression in RS4;11 cells transfected with pSG5 vectors encoding wild-type FHIT. **B.** Relative percentage of Annexin V positive RS4;11 cells after transfection with pSG5-FHIT or pSG5 (empty vector) at indicated time points as determined by flow cytometry. Error bars are from two independent experiments.

Ectopic FHIT expression in RS4;11 cells

Finally, we studied whether ectopic *FHIT* expression would confirm tumor suppressor activity in *MLL* rearranged ALL cells lacking endogenous *FHIT* expression as a conse-

quence of 5'CpG methylation. For this, RS4;11 cells were transfected with the eukaryotic expression vector pSG5 encoding wild-type *FHIT* or empty vector. Accordingly, the amount of cell death induced in RS4;11 cells transfected with either pSG5-FHIT or empty vector was expressed as a relative measure to control cells subjected to electroporation only. Interestingly, re-introduction of *FHIT* expression (Figure 8a) in RS4;11 cells clearly induced cell death as compared to cells transfected with empty vector only (Figure 8b). The transfection efficiency in RS4;11 cells (viable after electroporation) typically ranges between 40-45%. Thus, when corrected for the fact that approximately half of the cells actually were transfected with the pSG5-FHIT vector, the effects of *FHIT* re-expression in these cells may even be more pronounced.

DISCUSSION

MLL gene rearranged ALL (MLL) represents a unique leukemia predominantly occurring in children less than one year of age (i.e. infants). Infant MLL is an aggressive type of leukemia characterized by exceptionally high white blood cell (WBC) counts at presentation, resistance to multiple chemotherapeutic drugs[3,5] and a poor treatment outcome.[1,2] Because these patients often relapse on current therapies, a better understanding of this disease is warranted in order to develop more specific and successful treatment strategies. Here we report that infant MLL is characterized by silencing of the putative tumor suppressor gene *FHIT* as a consequence of 5'CpG island hypermethylation in 100% of the cases.

Analysis of our gene expression profiling data revealed *FHIT* expression to be reduced in patients with MLL when compared to other ALL patients.[8] Recently a similar observation has been reported[28]. In the present study we confirmed by quantitative real-time PCR that *FHIT* mRNA expression in infants with MLL is 11 to 15-fold lower as compared to both infant and non-infant ALL patients carrying germ line *MLL* genes, respectively. We found that the 5'CpG island region of *FHIT* is methylated in all 36 (100%) infant MLL cases tested, whereas in infant and non-infant ALL carrying germ line *MLL* genes this frequency was approximately 60%. Recently, Zheng *et al*[23] reported hypermethylation of the 5'CpG islands region of *FHIT* to be associated with hyperdiploid and translocation-negative subtypes of pediatric leukemia. In their study, the frequency of methylated FHIT was determined in childhood ALL patients positive for translocations t(12;21)/TEL-AML1, or other chromosomal rearrangements (not further specified) as well as in hyperdiploid positive and negative childhood ALL patients. The highest frequencies of methylated *FHIT* were observed in hyperdiploid positive (~22%) and in B-lineage ALL (~8%) and T-ALL (~38%) patients negative for TEL-AML1 or other translocations. The frequency of *FHIT* methylation in TEL-AML1

positive patients or patients positive for other translocations both appeared to be <1%.[23] Since translocations involving the *MLL* gene predominantly occur in infant ALL patients (less than 1 year of age), and these patients are under-represented in the study of Zheng and co-workers[23], the remarkably high frequency of *FHIT* 5'CpG methylation in patients harboring *MLL* rearrangements might therefore have been missed in that study.

The present study further demonstrates that *FHIT* methylation correlates with strongly reduced *FHIT* mRNA and FHIT protein expression. Moreover, reduced *FHIT* expression was confirmed to be a consequence of 5'CpG island methylation, as exposing leukemia cells carrying *MLL* gene rearrangements and methylated (silenced) *FHIT* to the demethylating drug 5'-aza-2'-deoxycytidine (decitabine) restores FHIT protein expression. Finally we demonstrate tumor suppressor activity of *FHIT*, as restoring the expression of this gene in RS4;11 cells in which FHIT is silenced by hypermethylation led to FHIT expression and induced leukemic cell death. This latter finding supports that the induced cell death as observed in SEMK2 cells upon exposure to decitabine, may at least in part be due to restored tumor suppressor activity of FHIT. Obviously, additional genes that become re-expressed upon demethylation may contribute to the observed toxicity to decitabine, especially since it was shown that *MLL* rearranged ALL cases display a high incidence of silenced genes that are known to be frequently methylated in hematological malignancies.[29] Therefore, treatment with demethylating agents such as decitabine, may particularly be effective in *MLL* rearranged leukemias. Supporting this hypothesis is our finding that decitabine (like other cytosine analogues) depend on the human equilibrative nucleoside transporter 1 (hENT1) to cross the cell membrane,[30] which is highly expressed in infant MLL cells.[25] Thus, compared to other ALL subtypes, demethylating drugs are likely to up-regulate higher numbers of silenced genes and may be transported more effectively into the leukemic cells from (infant) MLL patients.

For childhood leukemia a two-step mutation model has been proposed in which the first mutation occurs *in utero*, followed by a second, post-natal mutation giving rise to a clinically overt leukaemia.[31] This is, among other evidence, supported by the fact that fusion products generated by balanced translocations involving *MLL* can be identified on neonatal bloodspots of children developing leukemia within the first years of age.[32] Assuming that rearranged *MLL* is one of the initial events in the development of infant MLL, the relatively short latency period between this event and the emergence of overt leukemia suggests that possibly all genetic hits necessary for infant MLL to develop occur *in utero*. *FLT3* mutations may be secondary events,[9] however the incidence of these mutations in infant MLL is limited.[10,33,34] Our finding that the *FHIT* gene is silenced in all infant MLL cases tested, raises important questions; is loss of *FHIT* expression a secondary genetic hit additionally required for clonal expansion of (pre)neoplastic cells

that have acquired translocations involving *MLL*, or is 5'CpG island hypermethylation of *FHIT* rather a consequence of *MLL* gene rearrangements? Another possibility might be that methylated FHIT is an epigenetic phenomenon that fits the immunophenotype of highly immature B-cells characteristic for (infant) MLL.

Encompassing a common fragile site, *FHIT* is highly susceptible to genomic alterations like translocations and deletions upon exposure to environmental carcinogens (e.g. cigarette smoke and alcohol).[35] Interestingly, exposure to cigarette smoke has recently been associated with *FHIT* methylation in non-malignant lung tissue from heavy smokers.[26] However, parental smoking during pregnancy does not seem to directly contribute to the risk of childhood ALL.[36,37] Therefore the finding that the frequency of *FHIT* 5'CpG methylation is considerably higher in infant ALL patients carrying translocations involving MLL as compared to *MLL* germ line infant ALL patients rather implies that *FHIT* methylation may be driven by the *MLL* fusion protein itself. This hypothesis is supported by a recent study showing that the leukemia promoting PML-RAR fusion protein in t(15:17) positive acute promyelocytic leukaemia (APL) binds to the promoter region of the putative tumor suppressor gene RARβ2 and subsequently recruits either DNMT1 or DNMT3 to hypermethylate the promoter.[38] Our finding that 100% of the infant MLL cases tested were positive for *FHIT* methylation may point to a similar mechanism. Especially since all MLL fusion proteins that arise from fusions between *MLL* and one of >30 different partner genes,[39] have in common that the *MLL* gene donates its N-terminal region containing the transcription repression domain. Part of this domain is highly homologues to the eukaryotic DNA methyltransferase 1 (DMNT1) and is able to specifically bind to unmethylated CpG sequences,[40] which may be critical in *MLL*-associated oncogenesis.[41] However, further studies are required to determine whether MLL fusion proteins are indeed responsible for gene silencing.

To conclude, the present study shows that (infant) MLL is uniformly characterized by silencing of the tumor suppressor gene FHIT as a consequence of 5'CpG hypermethylation and that restoration of the expression of this gene induces cell death in *MLL* rearranged ALL cells *in vitro*. This suggests that silencing of FHIT may be involved in leukemogenesis of this aggressive type of leukemia and may eventually be used as a novel therapeutic target.

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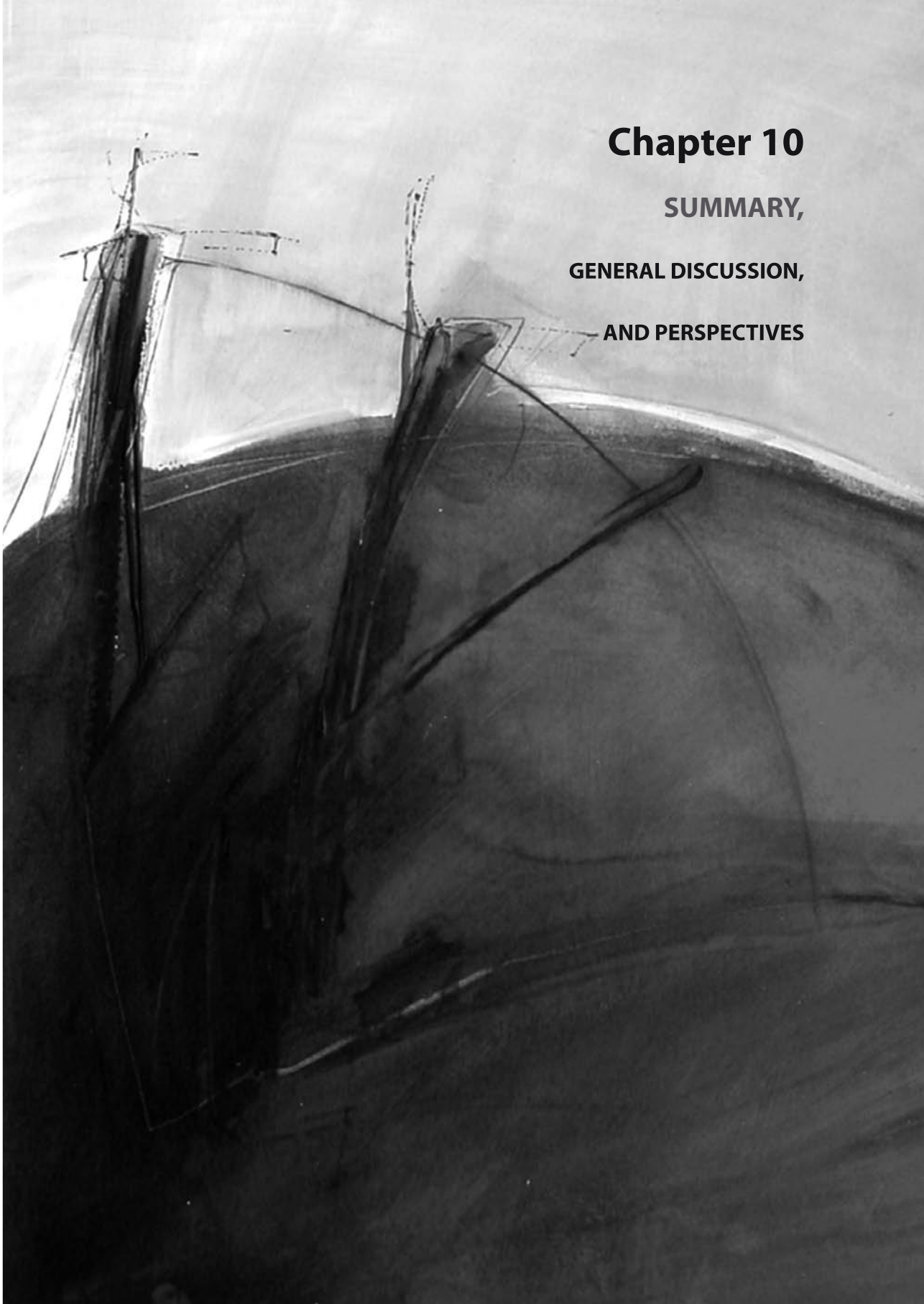

Chapter 10

SUMMARY,

GENERAL DISCUSSION,

AND PERSPECTIVES

Acute lymphoblastic leukemia (ALL) in infants is characterized by a high incidence (~80%) of rearrangements of the *MLL* gene, resistance to several important chemotherapeutic drugs, and a poor treatment outcome. The current literature and knowledge on infant ALL is extensively reviewed in **chapter 2**.

With overall survival rates for infant ALL not exceeding 50%, combination chemotherapy including glucocorticoids (prednisone and dexamethasone), vincristine, L-asparaginase, 6-mercaptopurine, methotrexate, and anthracyclines (daunorubicin and doxorubicin) as successfully used for the treatment of childhood ALL, obviously is not sufficient to treat ALL in infants. In 1998 we observed that, compared to cells from older children, leukemic cells from infant ALL patients are significantly more sensitive to the cytidine analogue Ara-C (cytosine arabinoside or cytarabine),[1] which is a drug typically used in the treatment of acute myeloid leukemia (AML). In 1999 this knowledge was implemented in an international collaborative treatment protocol for infant ALL, i.e. INTERFANT-99, in which the use of Ara-C is markedly intensified throughout the protocol.

As shown in **chapter 3**, Ara-C sensitivity in infant ALL cells appeared to be a result of elevated expression of the human equilibrative nucleoside transporter 1 (*hENT1*), on which Ara-C is mainly dependent to permeate the cell membrane.[2] Interestingly, sensitivity to Ara-C in infant ALL appeared not to be associated with rearrangements of the *MLL* gene, as both *MLL* rearranged and *MLL* germ line infant ALL cases appeared equally sensitive to this drug *in vitro*, and comparable *ENT1* expression levels were observed between both patient groups (**chapter 4**). Thus, both *MLL* rearranged and *MLL* germ line infant ALL patients may benefit from treatment regimens that include Ara-C. However, high-dose Ara-C regimens generate high extracellular drug concentrations *in vivo*, causing Ara-C also to enter the cell by passive diffusion. In other words, high-dose Ara-C circumvents membrane transport via ENT1. Therefore, elevated *ENT1* expression in leukemic cells from infants with ALL may only be beneficial when these patients are treated with low to moderate dosages of Ara-C. Nevertheless, improved outcomes have been reported for infant ALL patients treated with protocols in which high-dose Ara-C had been implemented during the consolidation phase.[3,4] Moreover, improved outcome for adult pro-B ALL cases (both *MLL* rearranged and *MLL* germ line) was noted with intensified post-remission therapy including high-dose Ara-C.[5] The INTERFANT-99 treatment protocol includes both low and high-dose Ara-C throughout the duration of the treatment, and therefore may well improve prognosis for infant ALL patients treated according to this protocol.

In addition to sensitivity to Ara-C, infant ALL cells also appeared to be highly sensitivity to the adenosine analogue 2-CdA (2-chlorodeoxyadenosine or cladribine).[6] Whether 2-CdA sensitivity in infant ALL can also be attributed to increased ENT1 expression remains uncertain. Wright, *et al* (2002) showed that 2-CdA does not seem

to enter pediatric ALL cells via the same membrane nucleoside transport system responsible for cellular Ara-C influx.[7] However, the number of ALL samples used in that study was rather small. In contrast, in **chapter 5** we demonstrate a significant correlation between *ENT1* expression and sensitivity to 2-CdA in a large cohort of childhood AML patients. Interestingly, in several studies synergistic effects between Ara-C and 2-CdA have been observed *in vitro*,[8-10] and the addition of 2-CdA to Ara-C containing regimens have been shown to increase complete remission rates in AML.[11,12] Taken together these observations support that regimens combining the use of Ara-C and 2-CdA may potentially be beneficial for infant ALL patients. Moreover, given the apparent sensitivity of infant ALL cells to nucleoside analogues, the use of newly developed nucleoside analogous like for example clofarabine[13,14] and troxacitabine (troxatyl),[15,16] may be interesting candidate drugs for further testing in infant ALL.

Another specific class of nucleoside analogue drugs that may additionally be effective specifically against *MLL* rearranged infant ALL (infant MLL) cells are DNA demethylating cytidine analogues such as 5-azacytidine, 5-aza-2'-deoxycytidine (decitabine), or the recently identified agent zebularine.[17] Determining the extent of concurrent hypermethylation of E-cadherin, Dap-kinase, O^6MGMT, p73, p16, p15 and p14, Gutierrez, *et al*[18] showed that among several pediatric ALL subtypes, *MLL* rearranged cases had the highest methylation index (i.e. number methylated genes divided by the number of genes studied). Thus, MLL seems to be characterized by aberrant DNA hypermethylation. In concordance with this, we observed that the tumor suppressor gene *FHIT* is silenced by 5'CpG island methylation in 100% of the infant MLL cases tested, whereas silencing of this gene in older children with ALL carrying germ line *MLL* genes was observed in only ~50% of the cases (**chapter 9**). Furthermore we demonstrated that ectopic expression of *FHIT* in *MLL* rearranged RS4;11 cells that lack endogenous *FHIT* expression, induced leukemic cell death. Likewise, exposing MLL cells to the demethylating agent decitabine resulted in re-expression of FHIT protein and also induced apoptosis. Therefore, inhibition of DNA methylation may be an effective novel therapeutic strategy in the treatment of infant MLL, especially since we found that decitabine (like other cytosine analogues) depends on ENT1 to cross the cell membrane (**chapter 5**), which is highly expressed in infant ALL cells (**chapter 3**).

Nevertheless, since combinations of multiple chemotherapeutic drugs rather than single (types of) agents are required to cure acute leukemias, extending the drug repertoire against infant MLL with several nucleoside analogues probably is not sufficient to significantly improve the survival rate. Thus, in addition to exploring the use of effective nucleoside analogue drugs, additional innovative treatment strategies are needed that either overcome resistance to conventional drugs (like prednisone and L-asparaginase) or that involve novel agents that more effectively target infant MLL cells in alternative ways.

Overcoming drug resistance inevitably requires understanding of the mechanisms involved. The fact that infant MLL cells literally are resistant to multiple drugs, may suggest the involvement of multidrug resistance (MDR) proteins, which are specialized membrane pumps capable of trafficking multiple drugs out of the cell. However, as shown in **chapter 6**, drug resistance in infant ALL is not likely a consequence of increased drug efflux mediated by MDR pumps.

Although highly informative, studies like this exclude only limited numbers of possible explanations for drug resistance at a time. Fortunately, the completion of the Human Genome (HUGO) project and the rapidly advanced gene expression profiling technologies, nowadays allows comparisons of multiple patient groups for the expression of vast numbers of genes Recently, we demonstrated how such a gene expression profiling study can be a suitable approach in understanding drug resistance at a genetic level. Comparing gene expression patterns in childhood ALL patients either resistant or sensitive to prednisone, vincristine, L-asparaginase, and daunorubicin *in vitro*, we found 124 differentially expressed genes to be related to resistance to these drugs.[19] Moreover, this gene expression signature associated with drug resistance appeared to be highly predictive for clinical outcome. Interestingly, only three of the 124 differentially expressed genes had been associated with drug resistance before, indicating that the mechanisms underlying resistance to these drugs are complex and largely unknown. Validation studies now need to further reveal the exact causal mechanisms that are involved. Some of these genes may represent universal determinants of drug resistance that apply to all types of leukemia, including infant MLL. For example, one of the genes that appeared to be over-expressed in prednisone-resistant ALL cells was *MCL-1*,[19] an anti-apoptotic member of the BCL-2 family. Since infant MLL cells are highly resistant to prednisone[1,6] we determined *MCL-1* expression in a group of infant MLL samples using real-time quantitative PCR. Interestingly, we observed increased *MCL-1* expression in infant MLL samples as compared to samples from older children with ALL, and found the expression of this gene to correlate with resistance to prednisone both in infant and non-infant ALL (Stam, *et al* unpublished data). MCL-1 plays an important role in the survival of multiple myeloma (MM) cells, in which *MCL-1* is abundantly expressed. Recently, two MCL-1 inhibitors, i.e. Seliciclib (CYC202 or R-roscovitine) and R-etodolac (SDX-101) were shown to induce apoptosis in MM cells by down-regulating MCL-1.[20,21] Moreover, sub-cytotoxic doses of R-etodolac sensitized MM cells to dexamethasone induced cell death. Therefore, prednisone resistance in infant MLL cells may possibly be overcome by compounds like Seliciclib and R-etodolac, or may directly induce leukemic cell death. Hence, inhibition of MCL-1 may be another therapeutic possibility to improve prognosis for infant MLL.

In addition to identifying genes possibly related to resistance to certain drugs, gene expression profiling techniques also are particularly suitable for the identification of

novel molecular therapeutic targets. As the comparison of gene expression profiles from drug sensitive and resistant patients may lead to the identification of genes involved in drug resistance, comparison of leukemia subtypes may identify genes that are uniquely expressed within certain types of leukemia, allowing the development of subtype specific therapy (i.e. targeted therapy). Several gene expression profiling studies have demonstrated that acute lymphoblastic leukemias carrying characteristic chromosomal abnormalities cluster together, clearly distinguishing themselves from other ALL subtypes.[22-24] Likewise, Armstrong, *et al* [25] demonstrated that *MLL* rearranged ALL specifies a unique type of leukemia displaying a gene expression pattern that is clearly distinguishable from both ALL and AML without *MLL* rearrangements. Soon after this finding, this MLL specific gene expression signature proved to be of great value for the discovery of novel therapeutic targets.

For example, *FLT3*, the gene encoding Fms-like tyrosine kinase 3, appeared to be one of the genes most consistently highly expressed in patients with MLL.[25] FLT3 is important in early B-lineage development[26] and as such is most abundantly expressed in immature B-cells,[27] which may explain the high expression of this gene in MLL cells, that typically display immature pro-B phenotypes. Normally FLT3 becomes activated upon binding of the hematopoietic growth factor FLT3 ligand (FLT3L). However, in AML the *FLT3* gene appeared to frequently carry mutations that constitutively activate this receptor in a ligand-independent manner, providing leukemic cells with a growth advantage and transforming capacity (reviewed by Gilliland and Griffin).[28] As such, constitutively activated FLT3 became a promising therapeutic target in AML. Several small molecule inhibitors have been shown to efficiently inactivate FLT3, accompanied by induced leukemic cell death *in vitro*. These findings prompted the initiation of several phase I/II clinical trials to determine the efficacy of these inhibitors against leukemic cells from refractory AML patients, and so far the results are promising.[29-31] Interestingly, we frequently observed constitutively activated FLT3 in primary samples from infants with MLL. However, screening these patients for the presence of known activating mutations revealed that such mutations rarely occur. Moreover, in an additional study in which we screened the entire *FLT3* gene for the presence of possible yet unidentified genetic abnormalities that may induce ligand-independent FLT3 activation, we found that it is unlikely that activated FLT3 in infant MLL is a result of activating mutations. In contrast, we observed that constitutively activated FLT3 in MLL patients frequently occurs in patients merely displaying high-level expression of wild-type *FLT3* (**chapter 7** and **chapter 8**). We (**chapter 7**) and others[32] have recently demonstrated that high-level *FLT3* expression in primary infant MLL samples is associated with activated FLT3 and cytotoxic responsiveness to FLT3 inhibitors. These data therefore show that FLT3 inhibition may represent a novel therapeutic strategy for infant MLL that urgently demands clinical testing.

Interestingly, in combination with Ara-C, some FLT3 inhibitors display synergistic cytotoxic effects in leukemic cells that are dependent on FLT3 activation.[33,34] This, together with the *in vitro* responsiveness of MLL cells to FLT3 inhibition, suggests that the addition of FLT3 inhibitors to Ara-C containing regimens may possibly further improve treatment response for patients with infant MLL.

In conclusion, infant MLL urgently requires innovative therapeutic strategies in order to improve prognosis. For this it is of utmost importance to understand this malignancy by accurately studying its unique molecular biological and properties. As shown in this thesis, this may provide insights into what type(s) of drugs may actually be effective against infant MLL, and may provide a sense for why other classes of drugs are highly ineffective. Moreover, this may lead to identifying genes specifically expressed in infant MLL that may be worth testing as therapeutic targets. Therefore, continued molecular studies designed to further gain insight in the biology of infant MLL, should ultimately lead to the development of effective treatment regimens, turning this aggressive type of leukemia into a curable disease, as has been reached for older children with ALL over the last decades.

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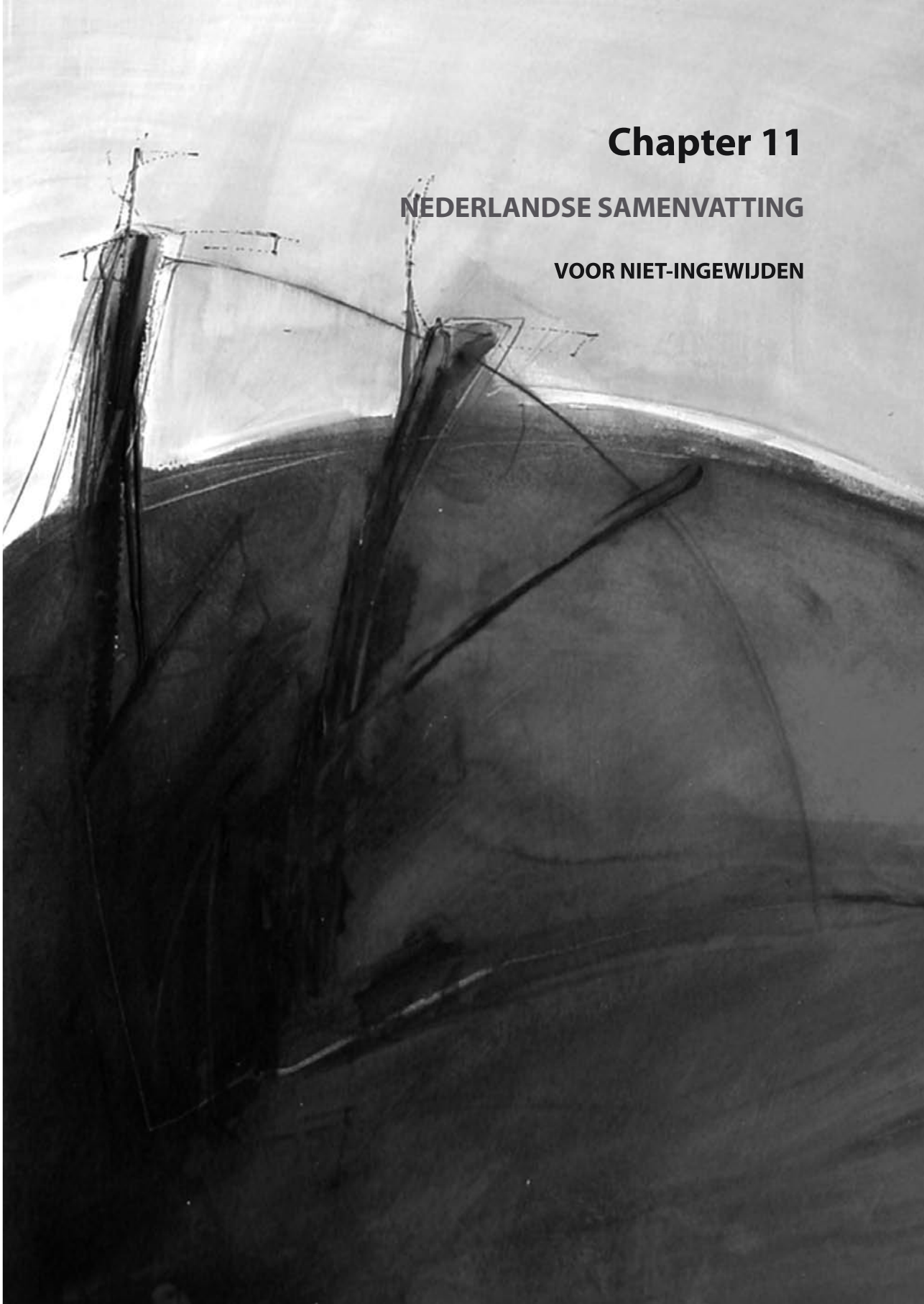

Chapter 11

NEDERLANDSE SAMENVATTING

VOOR NIET-INGEWIJDEN

BLOED

Ons bloed bestaat voor ongeveer de helft uit een gelige vloeistof (het bloedplasma), met daarin de verschillende soorten bloedcellen, waarvan de rode bloedcellen (welke het bloed de rode kleur geven) in aantal veruit het sterkst vertegenwoordigd zijn. Bloedcellen worden gevormd in een sponsachtig weefsel (het beenmerg) dat zich bevindt in de holten van onze botten, waarna ze worden afgegeven aan het bloed. Om een gezonde balans tussen de verschillende soorten bloedcellen te handhaven, maakt het beenmerg dagelijks ongeveer 1.000.000.000.000 (één biljoen) nieuwe bloedcellen aan. Al onze verschillende bloedcellen hebben met elkaar gemeen dat ze ontstaan uit dezelfde voorloper cel, de zogenaamde hematopoetische stamcel. Onder invloed van complexe mechanismen in het beenmerg ontwikkelen deze onrijpe, nog niet-functionele stamcellen zich tot functionele witte bloedcellen (belangrijk voor de afweer tegen ziektekiemen), rode bloedcellen (nodig voor het transport van zuurstof door het gehele lichaam) of bloedplaatjes (betrokken bij de stolling van het bloed). Wat betreft de witte bloedcellen maken we onderscheid tussen lymfatische cellen (de B- en T-lymfocyten) en niet-lymfatische cellen (zoals monocyten en granulocyten).

WAT IS LEUKEMIE?

Bij leukemie (Grieks voor "wit bloed"), ofwel kanker van witte bloedcellen, is er sprake van een witte bloedcel welke tijdens zijn uitrijping in het beenmerg "ontspoort" en zich voortdurend ongecontroleerd begint te vermenigvuldigen. Als gevolg hiervan zal er in het beenmerg een opeenhoping plaatsvinden van talloze nog niet-functionele witte bloedcellen (de leukemiecellen), allen afkomstig van de onrijpe witte bloedcel die initieel "ontspoorde". Deze opeenhoping van niet-functionele bloedcellen belemmert vervolgens de aanmaak van gezonde bloedcellen, hetgeen leidt tot bloedarmoede (door een tekort aan rode bloedcellen), bloedingen (door een tekort aan bloedplaatjes) en verhoogde gevoeligheid voor infecties (door een tekort aan gezonde witte bloedcellen). Uiteindelijk zullen de leukemiecellen ook terecht komen in de bloedsomloop, en van daaruit verschillende organen, zoals de milt, de lever en de nieren binnen dringen. Wanneer leukemie niet wordt behandeld is het een dodelijke ziekte.

VERSCHILLENDE TYPEN LEUKEMIE

Afhankelijk van het type witte bloedcel dat "ontspoorde" kan er onderscheid gemaakt worden tussen lymfatische en niet-lymfatische (of myeloïde) leukemie. Lymfatische

leukemie kan vervolgens weer worden onderverdeeld in B- en T-cel leukemie. Volgens hetzelfde principe wordt ook niet-lymfatische leukemie onderverdeeld in verschillende typen, afhankelijk van het type bloedcel dat is ontspoord. Lymfatische en niet-lymfatische leukemie worden vervolgens weer verder onderverdeeld in *chronische* of *acute* leukemie. Acute leukemie ontwikkelt zich zeer snel en agressief, terwijl chronische leukemie zich veel langzamer ontwikkelt.

LEUKEMIE BIJ KINDEREN

In Nederland wordt er jaarlijks bij ±150 kinderen leukemie geconstateerd. Daarmee is leukemie de meest voorkomende vorm van kanker op de kinderleeftijd, en tevens de belangrijkste doodsoorzaak van ziekte bij kinderen. Veruit het meest voorkomende type leukemie bij kinderen is acute lymfatische leukemie (ALL), welke wordt vastgesteld bij ongeveer 80% van alle kinderen met leukemie. Dankzij intensief onderzoek in de afgelopen decennia is de prognose voor kinderen met ALL heden ten dage relatief gunstig. Met behulp van combinatie chemotherapie geneest ongeveer 80% van alle kinderen met ALL. Echter, er zijn nog steeds kinderen met ALL waarvoor de prognose veel minder gunstig is.

LEUKEMIE BIJ ZUIGELINGEN

Ongeveer 4% van alle kinderen waarbij ALL wordt geconstateerd zijn jonger dan 1 jaar (zuigelingen). Met overlevingskansen variërend van ongeveer 40-50% is de prognose voor zuigelingen met ALL ongunstig te noemen. Naast een slechte prognose, laat ALL bij zuigelingen zich karakteriseren door het zeer frequent voorkomen (~80% van de gevallen) van chromosomale afwijkingen waarbij het zogenaamde *MLL* gen betrokken is. Bij oudere kinderen met ALL komen dit soort afwijkingen slechts zeer sporadisch voor. Deze afwijkingen in het *MLL* gen komen overigens alleen voor in de leukemiecellen, en niet in de gezonde cellen in het lichaam van de patiënt. Dit betekent dat deze gen afwijkingen dus niet erfelijk zijn, maar tijdens de ontwikkeling van het kind worden verworven. Verscheidene studies hebben aangetoond dat deze *MLL* afwijkingen tijdens de zwangerschap ontstaan in de witte bloedcellen van het nog ongeboren kind, en dat het tot stand komen van deze afwijkingen de eerste stap vormen van de transformatie van een gezonde witte bloedcel in een leukemiecel. Zuigelingen met ALL worden dan ook veelal geboren met leukemie, hoewel dat bij de geboorte nog niet echt merkbaar is. Verder is aangetoond dat de aanwezigheid van afwijkingen in het *MLL* gen in de leukemiecellen sterk geassocieerd is met een slechte prognose. Zuigelingen met ALL

waarbij deze afwijking niet gevonden wordt, hebben veel betere overlevingskansen. Het onderzoek beschreven in dit proefschrift richt zich dan ook specifiek op zuigelingen met ALL welke worden gekenmerkt door *MLL* afwijkingen, en dus een slechte prognose. **Hoofdstuk 2** bevat een gedetailleerde beschrijving van deze vorm van leukemie bij zuigelingen, en hoe de bevindingen uit het in dit proefschrift beschreven onderzoek mogelijk bij kunnen dragen aan een betere behandeling voor deze kinderen.

CELLULAIRE DRUG RESISTENTIE

Hoewel de huidige chemotherapeutische behandelingen zeer effectief zijn tegen ALL bij kinderen ouder dan 1 jaar, falen deze behandelingen in ongeveer 50% van de zuigelingen met ALL. Een belangrijke reden hiervoor is cellulaire chemotherapie resistentie. Leukemiecellen van zuigelingen met ALL zijn beduidend meer resistent voor enkele belangrijke medicijnen die deel uitmaken van de huidige behandeling van ALL, dan de leukemiecellen van oudere kinderen. Er is echter een uitzondering. Leukemiecellen van zuigelingen met ALL zijn zeer gevoelig voor het middel Ara-C, dat gewoonlijk gebruikt wordt voor de behandeling van acute myeloïde leukemie (AML).

GEVOELIGHEID VOOR ARA-C EN DE ENT1 TRANSPORTER

Ara-C is een medicijn dat als zodanig nog niet actief is, maar in de cel nog enige veranderingen moet ondergaan om uiteindelijk actief te worden. Eenmaal geactiveerd komt Ara-C in het DNA terecht, hetgeen resulteert in de vernietiging van de leukemiecel. Echter, alvorens het inactieve Ara-C in de cel omgezet kan worden in zijn actieve vorm, moet het eerst de cel binnen zien te komen. Als gevolg van de moleculaire structuur van Ara-C, kan dit geneesmiddel niet zomaar de leukemie cellen binnendringen, maar is hiervoor afhankelijk van gespecialiseerde eiwitten in de celmembraan (zogenaamde nucleoside transporters. Voor het transport van Ara-C over de cel membraan is hoofdzakelijk de nucleoside transporter ENT1 van belang. **Hoofdstuk 3** van dit proefschrift laat zien dat de leukemiecellen van zuigelingen met ALL (welke zeer gevoelig zijn voor Ara-C) beduidend meer ENT1 tot expressie brengen dan de leukemiecellen van oudere kinderen met ALL. Tevens blijkt de hoogte van de ENT1 expressie te correleren aan de gevoeligheid voor Ara-C. Met andere woorden, hoe meer ENT1 transporters er op een ALL cel zitten, hoe meer Ara-C de cel binnen kan dringen, en hoe meer leukemiecellen van deze patiënt door Ara-C vernietigd zullen worden. **Hoofdstuk 4** laat zien dat niet alleen zuigelingen met ALL en een *MLL* afwijking een hoge ENT1 expressie vertonen, en dus gevoelig zijn voor Ara-C, maar dat dit ook geldt voor zuigelingen

met ALL waarbij MLL afwijkingen ontbreken. In **Hoofdstuk 5** is aangetoond dat een verhoogde ENT1 expressie niet alleen gerelateerd is aan gevoeligheid voor Ara-C bij ALL patiënten, maar dat dit ook het geval is bij kinderen met AML. Dit laatste doet sterk vermoeden dat de snelheid waarmee Ara-C de leukemiecel binnen kan dringen via ENT1 (ofwel de hoogte van de ENT1 expressie) een algemene factor is die bijdraagt aan de gevoeligheid (bij hoge ENT1 expressie), of resistentie (bij lage ENT1 expressie) voor dit geneesmiddel. Echter, er is gebleken dat zeer hoge doseringen van Ara-C de leukemiecel binnen kunnen dringen zonder gebruik te maken van de ENT1 transporter. Nadeel hiervan is wel dat dit de kans op bijwerkingen van dit middel verhoogt. De bevindingen zoals beschreven in de boven genoemde hoofdstukken van dit proefschrift zouden er daarom toe kunnen leiden dat, wat Ara-C betreft, er in de toekomst "therapie op maat" gegeven kan worden. Kinderen met acute leukemie waarvan de leukemiecellen een hoge ENT1 expressie vertonen, kunnen bij voorkeur behandeld worden met lagere Ara-C doseringen. Gezien de gevoeligheid van deze patiënten voor Ara-C zullen afgepaste doseringen van dit middel afdoende moeten zijn voor het gewenste anti-leukemische effect, waarbij de kans op negatieve bijwerkingen beperkt blijft. Aan de andere kant zou ervoor gekozen kunnen worden om kinderen met acute leukemie waarvan de leukemiecellen een lage ENT1 expressie vertonen, te behandelen met andere medicijnen of, wanneer noodzakelijk, met hoge Ara-C doseringen.

MULITDRUG RESISTENTIE IN ALL BIJ ZUIGELINGEN

Naast het voorkomen van eiwitten in de celmembraan die bepaalde medicijnen helpen de leukemiecel binnen te dringen (zoals ENT1 dit doet met Ara-C), zijn er ook eiwitten in de cel membraan die precies het tegenovergestelde doen. Veel middelen die gebruikt worden in de behandeling van ALL bij kinderen vinden vrij eenvoudig hun weg de leukemiecel in. Vervolgens dienen deze middelen lang genoeg in de leukemiecel te verblijven om de leukemiecel te elimineren. In sommige gevallen bezitten leukemiecellen eiwitten op hun celmembraan die heel effectief allerlei medicijnen de cel weer uit kunnen pompen, nog voordat deze middelen hun dodelijke functie uit kunnen voeren. Omdat dit soort membraan transporters niet slechts één, maar vaak meerdere typen medicijnen (drugs) tegelijkertijd de cel uit kunnen pompen, worden deze transporters "multidrug resistentie eiwitten" genoemd. Het zou dus kunnen dat leukemie patiënten waarvan de leukemiecellen multidrug resistentie eiwitten bezitten veel moeilijker te behandelen zijn dan patiënten waarvan de leukemiecellen dit soort eiwitten niet bezitten. In **Hoofdstuk 6** van dit proefschrift is onderzocht of de expressie van een aantal bekende multidrug resistentie eiwitten bij zuigelingen met ALL (die resistent zijn voor veel medicijnen gebruikt in de behandeling van ALL) verhoogd zijn t.o.v. oudere

kinderen met ALL (welke over het algemeen gevoelig zijn voor medicijnen gebruikt in de behandeling van ALL). De resultaten van deze studie wijzen echter uit dat het onwaarschijnlijk is dat multidrug resistentie eiwitten een rol spelen bij de resistentie tegen chemotherapeutische middelen bij zuigelingen met ALL.

FLT3 REMMING ALS NIEUWE THERAPEUTISCHE BENADERING

Naast eiwitten in de cel membraan die allerlei medicijnen de cel in of uit transporteren, zijn er ook membraan eiwitten die fungeren als receptoren. Deze receptoren ontvangen signalen (in de vorm van moleculen) afgegeven door bijvoorbeeld omringende (vaak andere typen) cellen. Deze signaalmoleculen binden zich vervolgens aan hun receptoren, waarna de receptor het signaal weer doorgeeft aan de binnenzijde van de cel. De verwerking van zulke signalen heeft meestal tot gevolg dat de cel enige veranderingen ondergaat. FLT3 is zo'n membraan receptor. FLT3 speelt een belangrijke rol bij de uitrijping van B-lymfocyten. FLT3 komt dan ook met name tot expressie op de membranen van zeer onrijpe B-lymfocyten, en wordt geactiveerd door signaalmoleculen afkomstig van het beenmerg. Geactiveerd FLT3 spoort jonge, onrijpe B-lymfocyten aan om zich snel te vermenigvuldigen en beschermt ze tegen signalen die tot doel hebben de cel te vernietigen. Normaliter is de activering van FLT3 tijdelijk van aard en zal alleen plaatsvinden wanneer het nodig is, bijvoorbeeld als het lichaam behoefte heeft aan nieuwe, rijpe (en dus functionele) B-lymfocyten. **Hoofdstuk 7** en **Hoofdstuk 8** van dit proefschrift laten zien dat de leukemiecellen van zuigelingen met ALL welke *MLL* afwijkingen bezitten een zeer hoge expressie van FLT3 vertonen, en dat als gevolg hiervan de FLT3 receptor onafgebroken geactiveerd is, zelfs in de afwezigheid van geschikte signaalmoleculen. Hierdoor worden deze leukemiecellen voortdurend aangespoord om zich te vermenigvuldigen (één van de kenmerken van kankercellen) en worden ze beschermd tegen signalen die tot doel hebben de leukemiecel te vernietigen (zoals geïnduceerd door allerlei chemotherapeutische middelen). Dit zou dus heel goed kunnen bijdragen aan de verklaring waarom deze vorm van leukemie zo agressief is en moeilijk te behandelen. In **Hoofdstuk 7** is onderzocht of het middel PKC412, dat in staat is FLT3 te remmen, van therapeutisch belang kan zijn voor zuigelingen met ALL. Uit dit onderzoek bleek dat PKC412 inderdaad in staat is geactiveerd FLT3 op ALL cellen van zuigelingen met *MLL* afwijkingen te inactiveren, hetgeen gepaard gaat met de vernietiging van de leukemiecel. Het remmen van FLT3 zou daarom een effectieve nieuwe therapeutische benadering kunnen zijn voor zuigelingen met ALL.

INACTIVERING VAN FHIT IN ALL BIJ ZUIGELINGEN

In het algemeen wordt kanker veroorzaakt door de foutieve activering van genen die ongecontroleerde celdeling (vermeerdering) stimuleren (de zogenaamde proto-oncogenen) en/of de inactivering van genen die normaal gesproken helpen voorkomen dat een gezonde cel een kankercel wordt (de zogenaamde tumor suppressor genen). **Hoofdstuk 9** van dit proefschrift laat zien dat ALL bij zuigelingen gekarakteriseerd wordt door de inactivering van het tumor suppressor gen FHIT. Bij oudere kinderen met ALL komt de inactivering van FHIT veel minder frequent voor. Verder laat **Hoofdstuk 9** zien dat het re-activeren van FHIT in leukemiecellen van zuigelingen met ALL tot gevolg heeft dat de leukemiecel wordt vernietigd. Dit suggereert dat het inactief zijn van FHIT mogelijk betrokken is geweest bij het tot stand komen van de leukemie. Met andere woorden, om leukemiecel te kunnen worden heeft deze cel het FHIT gen uit moeten zetten; immers de activering van FHIT in deze cellen resulteert in celdood. Nu bestaan er verscheidene nieuwe medicijnen die in staat zijn geïnactiveerde genen te activeren. Zoals beschreven in **Hoofdstuk 9** re-activeren dit soort medicijnen ook geïnactiveerd FHIT. Een gunstige bijkomstigheid is dat dit soort middelen sterk lijken op het middel Ara-C, en daarom ook gebruik maken van de ENT1 transporter om de leukemiecel binnen te dringen (**Hoofdstuk 5**). Zoals beschreven in **Hoofdstuk 3** bezitten de leukemiecellen van zuigelingen met ALL zeer veel ENT1 transporters op hun membraan, waardoor dit soort middelen heel makkelijk de leukemiecel binnen kunnen komen. Het zou daarom heel goed kunnen dat deze medicijnen zeer effectief zijn voor de behandeling van ALL bij zuigelingen, hetgeen dan ook nader onderzocht zal worden in toekomstig onderzoek.

CONCLUSIES EN PERSPECTIEVEN

Om de prognose voor zuigelingen met ALL te verbeteren zijn er dringend nieuwe innovatieve behandel strategieën nodig. Teneinde dit te bereiken is het van groot belang dat we doorgaan met het bestuderen van de unieke moleculair biologische eigenschappen van deze agressieve vorm van leukemie. Zoals dit proefschrift laat zien, kunnen dit soort studies inzichten verschaffen in waarom sommige medicijnen niet werken, en welke (soort) medicijnen nu juist wel erg effectief zouden kunnen zijn. Het beschreven laboratorium onderzoek kan uiteindelijk leiden tot de identificatie van medicijnen die het waard zijn om daadwerkelijk bij patiënten te gaan testen, zoals bijvoorbeeld medicijnen die fungeren als FLT3 remmers. Nu is het onwaarschijnlijk dat een enkel medicijn op zichzelf voldoende is om de prognose voor zuigelingen met ALL drastisch te verbeteren. Echter, wanneer op deze manier meerdere medicijnen gevonden kunnen

worden die heel specifiek ingrijpen op verschillende biologische kenmerken van dit type leukemie, worden de mogelijkheden om tot een meer adequate behandeling te komen aanzienlijk vergroot. Resultaten voortvloeiend uit voortdurend laboratorium onderzoek zullen er dan ook toe moeten leiden dat ALL bij zuigelingen uiteindelijk een beter te genezen ziekte wordt, zoals dit in de afgelopen decennia bereikt is voor oudere kinderen met ALL.

ABOUT THE AUTHOR

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(Authored and co-authored by Ronald W. Stam)

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CURRICULUM VITAE

Ronald Wigle Stam werd geboren op 9 december 1974 te Vlaardingen. Van 1987 – 1992 bezocht hij de Christelijke Scholengemeenschap Het Groen van Prinsterer in Vlaardingen, alwaar hij in 1992 zijn HAVO diploma behaalde. Aansluitend ving hij aan met zijn laboratorium opleiding aan het Hoger Laboratorium Onderwijs (HLO) te Delft, waar hij in 1998 zijn diploma ontving in de studierichting Biochemie. Als onderdeel van deze studie liep hij stage op de afdeling Moleculaire Genetica aan het Nederlands Kanker Instituut (NKI/AvL) te Amsterdam. Onder de begeleiding van Dr. M. Snoek, verrichtte hij daar onderzoek naar longtumor gevoeligheidsgenen. Enthousiast geraakt voor het kankeronderzoek besloot hij in 1998 verder te studeren aan de Universiteit Leiden waar hij in 2000 zijn doctoraal in de Biologie behaalde.

Op 1 juli 2000 werd hij werkzaam als AIO op de afdeling kinderoncologie/hematologie aan het Erasmus MC – Sophia Kinderziekenhuis op het promotieonderzoek dat resulteerde in het tot stand komen van dit proefschrift. Onder de begeleiding van Prof. dr. Rob Pieters en Dr. M.L. den Boer verrichtte hij als promovendus onderzoek naar leukemie bij zuigelingen. In september 2005 ontving hij tijdens de 34ste SIOP (International Society of Pediatric Oncology) meeting de "SIOP award for basic science" voor het werk beschreven in één van de hoofdstukken van dit proefschrift.

Sinds juli 2004 is hij als wetenschappelijk onderzoeker werkzaam op de afdeling kinderoncologie/hematologie aan het Erasmus MC – Sophia Kinderziekenhuis, alwaar hij onder de supervisie van Prof.dr. R. Pieters momenteel vorm geeft aan zijn onderzoeksgroep, waarmee hij zijn onderzoek naar leukemie bij zuigelingen zal continueren.

DANKWOORD

"Dankbaarheid kan slechts voortkomen uit opgewekt innerlijk leven en uit een zekere vatbaarheid voor liefde."

Robert Saitschick

In de allereerste plaats wil ik diegene bedanken waar het in dit proefschrift echt om draait. Namelijk, kinderen met leukemie. Ik wil alle kinderen met leukemie, en hun ouders, die ertoe besloten hebben hun bloed en beenmerg te doneren als bijdrage aan het onderzoek naar deze afschuwelijke ziekte, vanuit de grond van mijn hart bedanken. Zonder jullie enorme bijdrage is het onderzoek naar leukemie eenvoudigweg niet mogelijk. Ik wil jullie bedanken dat ik onderzoek heb mogen doen op jullie leukemiecellen, en hoop dat ik daarmee mijn spreekwoordelijke steentje bij heb kunnen dragen, en nog lang bij zal mogen dragen, aan de bestrijding van deze uiterst oneerlijke, levensbedreigende ziekte bij kinderen.

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Zoals de mensen die mij de kans hebben gegeven onderzoek te doen naar leukemie bij zuigelingen. Te beginnen bij mijn promotor Prof.dr. R. Pieters. Beste Rob, enorm bedankt voor je prettige, ongecompliceerde en vaak gestroomlijnde (mt. vr. gr. r.) manier van begeleiden. Dank ook voor je ongeëvenaarde relativeringsvermogen en kristal heldere kijk op dingen. Om maar vooral alle invalshoeken te benutten, heb ik mezelf dikwijls doen verdwalen in complexe kluwen van gedachten. Kluwen waaruit jij vaak met speels gemak de essentie weer tevoorschijn haalde. Ook wil ik je bedanken voor het vertrouwen dat je altijd in me hebt gehad, getuige onder andere het feit dat je me de kans hebt gegeven m´n eigen onderzoeksgroep te vormen binnen het lab. Een kans die ik bijna had ontlopen, toen me de kans geboden werd m´n eigen laboratorium op te zetten op de afdeling kinderoncologie aan het Radboud ziekenhuis te Nijmegen. Ik hoop (en weet dat ook eigenlijk wel) dat je beseft dat je een belangrijk aandeel hebt gehad in mijn besluit in Rotterdam te blijven.

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Pauline (a.k.a. Plien, a.k.a. Mini-me, a.k.a. Hoofdzuster Oost-nederland, a.k.a. Die kleine, a.k.a. Hoofdzuster Amsterdam & omstreken), wat moet ik in vredesnaam zonder je? Onvermoeibaar en altijd even vrolijk pipetteer je al mijn hersenspinsels aan elkaar. Toen ik benaderd werd met het aanbod om m´n eigen lab op te zetten in Nijmegen, was je onvoorwaardelijk bereidt je met mij in dat grote avontuur te storten. Toen ik op het laatste moment besloot toch in Rotterdam te blijven, bleef je even onvoorwaardelijk achter me staan. Dat is me niet in de koude kleren gaan zitten. I owe you one! Niet alleen ben je een fijne collega en een prima analist, maar hebben Saskia en ik jou en Marien tevens mogen leren kennen als goede vrienden.

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Ronald